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# *In vitro* Assessment of Cisplatin and Oxaliplatin in Oesophageal Cancer

**Masters of Surgery (MS)**
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# Abstract

Oesophageal carcinoma is a common and highly virulent malignancy. Systemic metastatic disease is present in 50% of patients at diagnosis and will develop in the vast majority of the rest. Combined chemotherapy and radiotherapy is the standard regime of care in the non-surgical management of oesophageal cancer. Preoperative chemoradiotherapy followed by surgery continues to be actively studied in the surgical management of locally advanced oesophageal cancer. The limited efficacy and substantial toxicity of conventional 5-FU–cisplatin-based chemotherapy combined with radiation, has prompted the evaluation of newer agents, including the oxaliplatin, taxanes and irinotecan.

Oxaliplatin has demonstrated antitumor activity against cisplatin resistant colon cancer both *in vitro* and *in vivo* and is now used in the chemotherapeutic treatment of metastatic colon and rectal cancer. Despite extensive study the precise mechanisms of action of oxaliplatin are yet to be fully elucidated. Much of the knowledge is based on the extrapolation of the findings for cisplatin and other 1,2 diaminocyclohexane ligand (DACH) compounds. Like cisplatin, oxaliplatin reacts with DNA and forms DNA adducts at the same sites as cisplatin. However, oxaliplatin is markedly less reactive with DNA and forms fewer adducts with DNA than equimolar cisplatin. Both react with cellular proteins; a possibly significant mechanism of their toxicity.

This thesis compares the cytotoxicity and pharmacology of oxaliplatin and cisplatin on two oesophageal cancer cell lines *in vitro.* Gene expression studies were performed to determine the predictive value of Excision Repair Cross Complementing 1(ERCC1), a DNA repair enzyme, as a potential marker of chemotherapeutic response or resistance of cisplatin and oxaliplatin in oesophageal adenocarcinoma cells.

## Declaration

This project was designed by me with the help of Dr K Sales, which was later approved by my supervisor Prof Winslet. All experiments in this project were carried out by me. Statistical work was carried out by me with the help of Dr Richard Morris, Reader in Medical Statistics & Epidemiology at UCL Medical School,

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**Key words**
Oesophageal Adenocarcinoma; Oesophageal Squamous Cell Carcinoma; Cisplatin; Oxaliplatin; DNA adducts; NER; ERCC1; XPA; Apoptosis; Bcl-2 family; Protein damage; Caspases

## Abbreviations

1,2 diaminocyclohexane ligand (DACH); fluorouracil (5-FU); single strands braks (SSB); double strand break (DSB); interstrand crosslinks (ICLs); DNA protein cross-links (DPC); Nucleotide excision repair (NER); Base excision repair (BER); Global genomic repair (GGR); Transcription-coupled repair(TCR); Excision Repair Cross-Complementing 1(ERCC1) ; Xeroderma Pigmentosum (XP); Cockayne syndrome (CS); RNA polymerase II (RNAP-II); High-mobility groups (HMG); Thiordoxin (TRX), Buthionine sulphoximine (BSO); Glutathione synthase (GS); Time dependent index (TDI); Ribo nuclide acid (RNA); Messenger RNAs (mRNAs); Reverse transcriptase polymerase chain reactions (RT-PCR); Glyceraldehyde-3-phosphate dehydrogenase (GAPDH); Non-small-cell lung- cancer (NSCLC); Small-cell lung cancer (SCL); Mismatch repair (MMR) system; Thiordoxin (TRX); Roswell Park Memorial Institute (RPMI); phosphatidylserine (PS); phosphatidylserine receptor (PSR); Caenorhabditis elegans (C. elegans); Voltage-dependent anion channel (VDAC); Reactive oxygen species (ROS); Permeability transition (PT); ATP; Endoplasmic reticulum (ER); Mitochondrial outer membrane (MOM); Intermembrane space (IMS); Permeability transition pores (PTP); Poly (ADP-ribose) polymerase-1 (PARP-1); Nicotinamide adenine dinucleotide (NAD); Tumour necrosis factor (TNF); Death domain (DD); Fas Associated Death Domain (FADD); Death effecter domain (DED); Death Inducing Signalling Complex (DISC); TRADD; Inhibitor of apoptosis proteins (IAP); Apoptosis-inducing factor (AIF); Death-receptor (DR)

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# Chapter 1

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# Section I
## Oesophageal Carcinoma

### I.1 Introduction

Oesophageal cancer is a highly lethal disease, as reflected by an overall survival rate of 10–20% [1]. World-wide, almost 400 000 new patients are diagnosed annually and oesophageal cancer is the eighth most common cancer, and sixth on the list of cancer mortality causes [2]. In the UK, there are an estimated 7000 new diagnoses and 6700 deaths from carcinoma of the oesophagus every year [3, 4] .
Adenocarcinoma and squamous cell carcinomas are the commonest types of oesophageal cancers. Squamous cell carcinoma affects the upper two thirds of the oesophagus and adenocarcinoma affects the lower one third, but there are frequent exceptions to this rule [5-10]

Oesophagectomy has been regarded as standard treatment for patients with resectable oesophageal cancer [11,12]. This is based upon the concept that if all neoplastic tissue can be removed, a worthwhile period of survival and possibly a cure can be achieved [13]. Surgical resection is the only treatment that has repeatedly been shown to prolong survival with overall 5-year survival rates of 25%-35% [11,14, 15]. However, two thirds of patients will present with locally advanced T3-4 tumours, which are commonly associated with lymph node involvement (in up to 70%) and systemic metastases [16,17]. In addition, the majority of patients are elderly with co-morbid conditions who tolerate treatment poorly [18,19]. Therefore, potentially curative surgery is only possible for approximately 30% of patients [20]
An important determinant of surgical success and long-term survival is the clearance of all macroscopic and microscopic disease (termed as R0 resection) [21,22]. However, systemic relapse occurs in many patients even after R0 resections. Approximately 50% of patients will develop recurrent disease within one year of surgery and this has been attributed to the presence of clinically undetectable systemic micrometastases which are present at the time of diagnosis [11, 23,24]. Micrometastases have been demonstrated in rib marrow in 88% of patients undergoing curative surgery [25]. Thus surgery alone apprears non-curative and focus has been shifited to include multi-disciplinary approaches such as neoadjuvant chemotherapy, radiotherapy, chemoradiotherapy and post-operative radiotherapy and chemotherapy

## I.2 Rationale for neoadjuvant chemotherapy

In UK, Epirubicin/Cisplatin/5FU (ECF) or Mitomycin C/Cisplatin/5FU (MCF) combinations are used for adenocarcinoma and Cisplatin/5FU (CF) combination is used for squamous cell carcinoma of oesophagus [26]
The administration of chemotherapy prior to surgical resection has a number of theoretical and clinical advantages.[27] It has the potential to eliminate systemic micro-metastases as well as downstaging the primary tumour so that the likelihood of a curative R0 resection is increased and previously unresectable tumours become resectable [11]. Pre-operative chemotherapy has the advantage of delivering a higher concentration of chemotherapy agents to the tumour bed whilst its blood supply and lymphatics are still intact and prior to the tumour growth promoting stimulus of surgery [28]. Pre-operative therapy may devitalise tumour cells and minimise the risk of intraoperative spillage and seedling of viable tumour cells [29].

## I.3 Results of neoadjuvant chemotherapy

Most phase II and III trials currently use cisplatin-based combination chemotherapy. A recent review of cisplatin-based phase II trials for adenocarcinoma of the distal oesophagus reported major responses in 40-69% of patients, with pathological complete responses of 0-9% [29]. Resectability after pre-operative chemotherapy ranged between 58% and 96%. Toxicity was tolerable. Chemotherapy-related deaths were rare and operative mortality did not seem to be increased. The value of neoadjuvant chemotherapy has been further examined in a number of prospective randomised control trials (Table 1). However most of these trials randomised insufficient numbers and so had limited power to detect statistically significant differences of clinical importance. A further confounding factor is the lack of a standardised surgical approach. There is considerable debate about the extent of tumour resection and lymphadenectomy and routes of surgical access. This will be addressed in the forthcoming MRC OEO5 trial.

The two largest trials have produced conflicting results.The large intergroup trial INT0113 conducted by Kelsen and colleagues with 440 patients neither showed an improvement in the resectability nor in survival rates after induction chemotherapy [30]. Only a single large study from the Medical Research Council (MRC) including 802 patients suggested significantly improved survival after induction chemotherapy with cisplatin and 5-FU [31]. It was hypothesized that the shorter duration of chemotherapy in the UK trial leading to shorter delay of surgery may have

contributed to the favourable results. Importantly, patients in the MRC trial were treated more consequently according to the randomisation arm than in the INT0113 trial. A meta-analysis of eleven randomised trials by the Cochrane library revealed that pre-operative chemotherapy plus surgery appears to offer a potential survival advantage at 3, 4 and 5 years. However, significance was reached only at 5 years compared to surgery alone for resectable thoracic oesophageal cancer of any histological type [32] An ongoing trial (MRC-OE 05/EU- 20204) is investigating the addition of epirubicin to cisplatin plus fluorouracil vs. cisplatin and fluorouracil alone in the neo-adjuvant treatment of patients with adenocarcinoma of the oesophagus [33]

Table 1 Oesophageal carcinoma and pre-operative chemotherapy: randomised phase III trials

| Study | Patients | Therapy | CR + PR (%) | Resectability rate (%) | pCR | Post-operative mortality (%) | Median survival (m) | 3-year survival (%) |
|---|---|---|---|---|---|---|---|---|
| Roth 1988 | 39 | Sx | | n.a. | | 0 | 9 | 5 |
| | | CT + Sx 1,2,3 | 47 | n.a. | n.a. | 12 | 9 | 25 |
| Nygaard 1992 | 91 | Sx | | 69 | | 13 | n.a. | 9 |
| | | CT + Sx 1,3 | n.a. | 58 | n.a. | 15 | n.a. | 3 |
| Schlag 1992 | 46 | Sx | | 79 | | 10 | 10 | n.a. |
| | | CT + Sx 1,4 | 50 | 70 | 9 | 19 | 10 | n.a. |
| Maipang 1994 | 46 | Sx | | n.a. | | n.a. | 17 | 36 |
| | | CT + Sx 1,2,3 | 53 | n.a. | 13 | n.a. | 17 | 33 |
| Kok 1996 | 148 | Sx | | 85 | | | 11 | n.a. |
| | | CT + Sx 1,6 | 36 | 85 | n.a. | n.a. | 19 | n.a. |
| Kelsen 1998 | 440 | Sx | | 89 | | 6 | 16 | 23 |
| | | CT + Sx 1,4 | 19 | 76 | 2.5 | 6 | 15 | 26 |
| MRC 2002 | 802 | Sx | | 70 | | 10 | 13.3 | 34* |
| | | CT + Sx 1,4 | n.a. | 78 ($p = 0.001$) | | 11 | 17.2 | 43* ($p = 0.003$) |
| Law 1997 | 147 | Sx | | 35 | | 8.7 | 13 | 44* |
| | | CT + Sx 1,4 | 58 | 67 | 6.7 | 8.3 | 16.8 (NS) | 31* ($p = 0.13$) |
| Baba 2000 | 42 | Sx | | 83.3 | | 0 | n.a. | 75# (T1,2), 15# (T3) |
| | | CT + Sx 1,4,LV | 60 | (both groups) | 0 | 4.7 | n.a. | 57# (T1,2), 36# (T3) |
| Wang 2001 | 100 | Sx | | | | | | 32# |
| | | CT + Sx | n.a. | | | | | 46# |
| Ancona 2001 | 96 | Sx | | 74.4 | | 4.2 | ca. 29 | 22# |
| | | CT + Sx 1,4 | 40 | 78.7 | 12.8 | 4.2 | ca. 29 | 34# ($p$ = n.s.) |

Sx = surgery, C = chemotherapy, n.a. = not available. *2-year survival, #5-year survival, CR + PR = complete and partial remission, pCR = pathologically complete remission. 1 = cisplatin, 2 = vindesin, 3 = bleomycin, 4 = 5-FU, 5 = vinblastin, 6 = etoposid.

The MAGIC trial has recently reported on the use of neoadjuvant combination of epirubicin, cisplatin and 5-fluorouracil (ECF) chemotherapy in operable gastric and lower oesophageal adenocarcinoma [34]. This chemotherapy combination is currently regarded as the gold standard for advanced oesophago-gastric cancer in the UK [35,36]. Between 1994 and 2002, 503 patients (74% gastric, 15% gastro-oesophageal junction and 11% oesophageal) were randomised to receive perioperative chemotherapy with ECF or surgery alone. The results show a significantly better disease-free survival with the use of perioperative chemotherapy. Overall survival at two years was 48% in the perioperative chemotherapy arm and 40% in the surgery alone arm. Significantly more patients underwent a curative resection with perioperative chemotherapy [37].

The lack of a definitive answer to the role of neoadjuvant chemotherapy from these randomised controlled trials has been addressed by a number of recent meta-analyses (Table 2). These have consistently reported little or no survival advantage for the use of neoadjuvant chemotherapy in operable oesophageal cancer. Meta-analysis methodology varied. When only the more recent randomised controlled trials that combined cisplatin and 5-fluorouracil were used in the meta-analysis, the survival advantage for neoadjuvant chemotherapy at two years became more marked. [14]. However, separating out the two trials that included post-operative chemotherapy in addition to pre-operative chemotherapy failed to show any survival benefit [30,38]. A meta-analysis of eleven randomised trials by the Cochrane library revealed that for adult patients with resectable thoracic oesophageal cancer for whom surgery is considered appropriate, surgery alone without neoadjuvant treatment should be regarded as standard treatment [24].

Table 2 Summary of Meta-analysis of neoadjuvant chemotherapy

| TABLE 2. SUMMARY OF REPORTED META-ANALYSES OF NEOADJUVANT CHEMOTHERAPY FOR OESOPHAGEAL CANCER | | | | |
|---|---|---|---|---|
| **Author** | **Year** | **RCTs** | **Patients** | **Main Outcomes** |
| **Urschel *et al*[7]** | 2002 | 11 | 1976 | Neoadjuvant chemotherapy significantly associated with lower overall rate of resection (OR=1.71, 95% CI=1.22-2.40, p=0.002) but higher rate of R0 resection (OR=0.71, 95% CI=0.58-0.87, p=0.001)<br>No significant difference in 1 and 2 year survival, patterns of recurrence, perioperative morbidity or mortality |
| **Malthaner and Fenlon[8]** | 2002 | 7 | 1653 | No siginificant difference in 1 year survival (OR=1.03, 95% CI=0.78-1.36)<br>20% significant decrease in mortality at 2 years (OR=0.80, 95% CI=0.65-0.99) with a fixed effects model only |
| **Kaklamanos *et al*[9]** | 2003 | 7 | 1683 | 4.4% 2 year survival advantage for neoadjuvant chemotherapy (95% CI=0.3-8.5%)<br>1.7% increase in treatment-related mortality compared with surgery alone (95% CI=0.9-4.3%) |
| **Malthaner *et al*[10]** | 2004 | 7 | 1401 | No siginificant difference in risk of mortality at 1 year (RR=1.00, 95% CI=0.83-1.19, p=0.98) |

## I.4 Pre-operative radiotherapy

Five randomised studies failed to show a clear advantage for pre-operative radiotherapy plus surgery compared with surgery alone [39] (Table 3). Unfortunately, fractionation schemes and total doses for pre-operative schedules varied considerably from 20 to 40 Gy [40,41]. However, one large study revealed a reduced local relapse rate after preoperative radiotherapy (RT) with 33 Gy in 3.3 Gy fractions [42] and another trial showed a borderline survival advantage with 35 Gy in 20 fractions [43] (Table 3). Optimal techniques may harbour a potential for improving on RT results in the pre-operative setting. At present, RT followed by surgery is not considered to be a better treatment than chemo-radiation or surgery alone, and should not be recommended outside of controlled studies.

Table 3 Oesophageal carcinoma and pre-operative radiotherapy: randomised phase III trials

| Study | Treatment Patients arm | | Radiation dose (Gy) | Resectability rate (%) | Post-operative mortality (%) | Local recurrence (%) | 5-year survival (%) |
|---|---|---|---|---|---|---|---|
| Launois 1981 | Sx | 57 | | 70 | 23 | n.a. | 12 |
| | RT + Sx | 62 | 40 | 76 | 23 | n.a. | 10 |
| Gignoux 1987 | Sx | 106 | | 82 | 18 | 67 | 9 |
| | RT + Sx | 102 | 33 | 74 | 17 | 46° | 10 |
| Mei 1989 | Sx | 102 | | 85 | 6 | 12 | 30 |
| | RT + Sx | 104 | 40 | 93 | 5 | 13 | 35 |
| Arnott 1992 | Sx | 86 | | 72 | 13 | n.a. | 17 |
| | RT + Sx | 90 | 20 | 74 | 15 | n.a. | 9 |
| Nygaard 1992 | Sx | 41 | | 69 | 13 | n.a. | 9* |
| | RT + Sx | 48 | 35 | 54 | 11 | n.a. | 21* ($p = 0.08$) |
| Wang 1989 | Sx | 102 | | 85 | 6 | 4 | 30 |
| | RT + Sx | 104 | 40 | 93 | 5 | 13 | 35 |

*3-year survival (%), °$p = 0.009$, Sx = surgery, RT = radiotherapy, n.a. = not available.

## I.5 Pre-operative chemo-radiation

Considerable concern exists whether pre-operative chemo-radiation should be accepted as a standard in multimodal treatment of locally advanced oesophageal carcinoma because only one debatable trial showed a clear survival benefit compared with surgery alone [44] (Table 4). In two meta-analysis, a significantly improved 3-year survival rate was found only if pre-treatment was associated with downsizing [45,46]. Notably, the superior results of pre-operative chemo-radiation are due to the responses in patients with adenocarcinoma whereas long-term survival is not improved for patients with squamous cell carcinoma [46].

Pre-operative chemo-radiation did not improve the 1- and 2- year survival rates, most probably because of the peri-operative mortality [45]. An important endpoint is the survival rate at three years. Pre-operative chemo-radiation offers a definitive advantage to surgery alone with regard to the meta-analysis by Fiorica and collegues and by Urschel and colleagues [45,46]. This advantage has to be balanced to the toxicity associated with chemo-radiation. The timing of chemotherapy and RT is noteworthy and may be important for optimal therapeutic efficacy. Out of eight studies five used concomitant chemo-radiation [47–52] while in three, a sequential [44, 52,53] treatment schedule was used. Walsh and colleagues used concomitant chemoradiation for the treatment of oesophageal adenocarcinoma followed by surgery eight weeks later. Compared to surgery alone, median and overall survival was improved [48]. However, this study was criticized because the survival in the surgery alone arm was low. In a similar although underpowered study by Urba and colleagues a better outcome for the patients treated with pre-operative chemo-radiation was reported initially, but the final publication revealed no significant benefit for chemo-radiation [47,54]. In these two studies, adenocarcinoma was present in about 100% and 75%, respectively. A trend for improved survival was observed in the study reported by Apinop and colleagues [49].

Table 4 Oesophageal carcinoma and pre-operative chemo-radiation: randomised phase III trials

| Study | Patients (*n*) | Stage | Therapy | Resectability rate (%) | pCR (%) | Post-operative mortality (%) | Median survival (months) | 3-year survival (%) |
|---|---|---|---|---|---|---|---|---|
| *Concomitant* | | | | | | | | |
| Apinop 1994 | 67 | IIb-III | Sx alone | 100 | | 15 | 7.4 | 10° |
| | | | CRT (1,3/40 Gy) + Sx | 74 | n.a. | 14 | 9.7 | 24°/*p* = n.s. |
| Urba 2001 | 100 | T1-4*, N0-1 | Sx alone | 90 | | n.a. | 17.6 | 16 |
| | | | CRT (1,3/45 Gy) + Sx | 90 | 28 | n.a. | 16.9 | 30/*p* = 0.15 |
| Walsh 1996 | 113 | n.a. | Sx alone | n.a. | | 4 | 11 | 6 |
| | | | CRT (1,3/40 Gy) + Sx | n.a. | 25 | 9 | 16 | 32/*p* = 0.006 |
| Burmeister '02 (abstract only) | 256 | n.a. | Sx alone | n.a. | | 4.6 | 18.5 | n.a. |
| | | | CRT (1,3/35 Gy) + Sx | n.a. | 15.1 | 4.6 | 21.7/*p* = 0.38 | n.a. |
| Lee 2003 (abstract only) | 102 | II, III | Sx alone | n.a. | | n.a. | 27.3 | n.a. |
| | | | CRT (1,3/45.6 Gy bid) + Sx | n.a. | 43 | n.a. | 28.2/*p* = 0.67 | n.a. |
| *Sequential* | | | | | | | | |
| Bosset 1997 | 282 | T1-3, N0-1 | Sx alone | >80 | | 4 | 18.6 | 32 |
| | | | CRT (1#/37 Gy) + Sx | >80 | 26 | 12 | 18.6 | 32 |
| Nygaard 1992 | 103 | T1-2, NxM0 | Sx alone | 69 | | 13 | n.a. | 9 |
| | | | CRT (1,2/35 Gy) + Sx | 66 | n.a. | 24 | n.a. | 17/*p* = 0.3 |
| LePrise 1994 | 86 | T1-3 | Sx alone | 84 | | 7 | 10 | 13.8 |
| | | | CRT (1,3/20 Gy) + Sx | 85 | n.a. | 9 | 10 | 19.2/*p* = n.s. |

1 = cisplatin, 2 = bleomycin, 3 = 5-FU, *exclusion of M+ and one tumor, that required laryngectomy, °5-year-survival, #0–2 days before each cycle of radiotherapy, pCR = pathologically complete remission, n.a. = not available, n.s. = not significant, CRT = chemo-radiation, Sx = surgery.

The large trial by Bosset and colleagues on behalf of the European Organization of Radiotherapy of Cancer (EORTC) used sequential pre-operative chemo-radiation in squamous cell carcinoma of the thoracic oesophagus [51]. The overall survival was similar for both treatment arms, although the disease-free survival was improved in patients treated with chemo-radiation ($p = 0.003$). Currently, the FFCD-9901/EORTC-22001 trial is investigating neo-adjuvant chemo-radiation (continuous fluorouracil and cisplatin) with surgery vs. surgery alone in patients with thoracic oesophageal carcinoma [55].

Taking into account all the experience with chemo-radiation, results with concomitant schedules seem to be more potent than schedules using sequential chemo-radiation. Clearly, one major rational for pre-operative cytotoxic treatment is the ability to achieve downstaging and thus improving on the chances of radical surgery. Using endosonography, downstaging can be detected in about 30% [48] to 60% [56]. Even complete remission after chemo-radiation can be achieved and is seen in about 21% [45] to 30% [57] or 38% [58] of patients. Histopathological complete remission is more likely observed after concomitant chemo-radiation. Good response to induction treatment and the pathologic stage following neo-adjuvant chemo-radiation are valid prognostic factors. Most interestingly, recent studies suggested, that in case of pathological complete remission surgery may even be omitted [59, 60].

## I.6 Definitive chemo-radiation

Currently, definitive chemo-radiation is a standard treatment for non-surgical patients if co-morbidity prohibits surgery or if the extension of the disease is judged to be primarily non-resectable. In such patients, a 2-year survival rate of 38% can be achieved with chemo-radiation alone as shown in two large randomised phase III trials [61–63] (Table 5). Concomitant chemo-radiation provides significant overall reduction in mortality, and reduces the mortality rate from 62% to 55% and from 83% to 76% after 1 and 2 years, respectively [64]. Unfortunately, the local recurrence rate is only reduced from 68% to 56% and toxicity significantly increased indicating the need for more potent and less toxic radiation sensitizers or novel antitumor drugs. A recent Japanese trial comparing late-course accelerated hyperfractionated RT with or without concomitant cisplatin however failed to show a significant improvement in outcome [65].

In locally advanced operable oesophageal cancers responding to chemo-radiation definitive chemo-radiation was an alternative to surgery, because overall survival was

equal and early mortality including length of hospital care was better with chemo-radiation alone [59]. Palliation procedures for dysphagia were more frequent after chemo-radiation than surgery. Another randomised phase III study comparing definitive chemo-radiation with pre-operative chemo-radiation showed no improved 3-year survival (50%) for the three-modality treatment arm despite better local tumour control [60]. Additional phase III trials investigating the need for surgery after chemoradiation are required, including the assessment of predictive parameters in order to define a patient population that would not benefit from further surgery.

Table 5 Oesophageal carcinoma and definitive chemo-radiation: phase III trials

| Study | Patients (*n*) | Therapy | Mortality (%) | Local failure* (%) | Median survival (m) | 2-year survival (%) | 5-year survival (%) |
|---|---|---|---|---|---|---|---|
| Cooper, Herskovic, Al-Sarraf 1999 | 123 | RT (64 Gy) alone | 0 | 65 | 9.3 | 10 | 0 |
| | | CRT (50 Gy) | 4° | 54 | 14.1 | 36 | 27 |
| Minsky 2002 | 218 | CRT (50.4 Gy) | 2 | 55 | 18.1 | 40 | n.a. |
| | | CRT (64.8 Gy) | 10 | 50 | 13 | 31 | n.a. |
| Bedenne (abstract only) 2002 | 259 | CRT alone | 1 | n.a. | 19.3 | 40 | n.a. |
| | | CRT + Sx | 9 | n.a. | 17.7 | 34 | n.a. |
| Stahl 2005 | 172 | CRT (60 Gy) | 3.5 | 59 (at 2y) | 14.9 | 35.4 | n.a. |
| | | CRT (40 Gy) + Sx | 12.8 | 35 (at 2y) | 16.4 | 39.9 | n.a. |

RT = radiotherapy, CRT = chemo-radiation, Sx = Surgery, *persistence or local recurrence, °2% radiotherapy-related, 2% chemotherapy-related, n.a. = not available.

## I.7 Post-operative radiotherapy

Post-operative adjuvant monomodality treatment after transthoracic or transhiatal resection does not improve outcome. Post-operative RT showed no benefit in three studies and might even increase the risk of post-operative mortality [66–68]. A more recent study by Xiao and colleagues although not randomised, reiterated the discussion whether or not post-operative RT should be considered in selected patients, reporting that patients with nodal involvement who tolerated post-operative RT showed an improved 5-year survival rate [69]. One reason why post-operative RT harbours major risks of toxicity is that correct oncological treatment demands coverage of all the tissue set at risk for microscopic contamination during surgery. In how far more focused and risk-adapted RT to specific anatomical areas such as the anastomosis could be beneficial remains unclear and cannot be recommended for patients treated outside clinical trials.

## I.8 Post-operative chemotherapy

Japanese groups investigated post-operative chemotherapy. One trial with post-operative cisplatin and fluorouracil suggested to prolong 5-year disease-free survival (45% vs. 55%) whereas 5-year survival was not improved (52% vs. 61%) [70]. An earlier trial of the same group also failed to show a positive effect using post-operative chemotherapy [71]. A comparison of post-operative chemotherapy with post-operative chemo-radiation was performed in Japan showing no difference in 1-, 3-, and 5-year survival [72] A Western CALGB- 80101-trial is currently randomising between adjuvant chemo-radiation after resection and surgery alone in patients with gastro-oesophageal adenocarcinoma or gastric cancer [73]

## I.9 Response evaluation for chemotherapy

Patients generally tolerate neoadjuvant chemotherapy better than post-operative (adjuvant) chemotherapy; only 38% and 40% of patients completed post-operative chemotherapy in the INT0113 and MAGIC trials respectively [30, 74, 75]. Furthermore, patients who respond to the treatment may have improved swallowing and nutritional baselines and performance status prior to surgery with a resultant decrease in post-operative morbidity and mortality [76].

Response to treatment is also important. A complete pathological response is defined as the absence of any residual microscopic evidence of oesophageal cancer

in the resection specimen after neoadjuvant treatment and is associated with a survival benefit [30, 60-62]. Pathological complete responses are seen in approximately 10% of patients after neoadjuvant chemotherapy. Current methods of response evaluation are based upon combinations of symptomatic, endoscopic and radiological assessments, usually performed after the completion of neoadjuvant treatment. To allow tailored management based upon response evaluation, these assessments need to be accurate, readily available, minimally invasive and safe.

Patients with responding tumours often show improvements in swallowing after only one cycle of chemotherapy. Validated scales exist for the measurement of dysphagia [77]. Weight gain during chemotherapy is associated with both tumour response and a survival benefit in both operable disease and advanced metastatic disease [78,79]. However, the absence of weight gain does not preclude a response and weight changes during chemotherapy may be affected by other factors such as chemotherapy toxicity.

The accuracy of endoscopic assessment of response to neoadjuvant therapy has been reported as 21%-59% [80-82]. Patients predicted endoscopically to have a pathological complete response and with subsequent histological confirmation had an excellent prognosis. If, however, a less than complete response was predicted endoscopically, survival was significantly worse. False positives may not represent a clinical problem; a false negative endoscopic assessment (seen in 27%) may deprive those patients with a pathological complete response or with only microscopic residual disease from further appropriate treatment if endoscopy alone is used to define response [37]

Radiological response evaluation is usually based upon applying World Health Organisation criteria to serial radiological imaging [78]. It is difficult to accurately measure carcinoma of the oesophagus in two dimensions and so clinical response monitoring is less standardised than for other tumours [79]. Both computed tomography (CT) and endoluminal ultrasound scanning have a reported accuracy of less than 50% in setting [81, 83-87]. The majority of inaccuracies result from an overestimation of tumour wall penetration. An alternative method is to measure changes in tumour size. When a 50% reduction in maximal cross-sectional area as measured by endoluminal ultrasound is used as the criterion for pathological response, the positive predictive value for detecting a pathological response is at least 75%.

Furthermore, responders based upon changes in tumour size have a significantly better survival than non-responders [88-90].

It is clear that changes in tissue metabolism within upper gastrointestinal cancers precede anatomical changes. This forms the basis of 18Fluorodeoxyglucose positron emission tomography (18FDG-PET) imaging. Studies evaluating tumour response with 18FDG-PET during and at the completion of neoadjuvant therapy have yielded encouraging results. These studies indicate that decreases in 18FDG uptake in response to neoadjuvant therapy correlate strongly with pathological response. Early prediction of response has the potential to differentiate non-responders from responders, minimise therapy toxicity and direct non-responders towards salvage or palliative therapies. However, many of these studies have small patient numbers and longer term follow-up is required.

A further advancement has been the development of integrated PET/CT imaging, which allows the contemporaneous correlation of the functional information of 18FDG-PET with the anatomical information of CT [91]. This has the potential to clarify both subtle metabolic findings from 18FDG-PET and equivocal CT findings by comparison with the other imaging modalities and may aid the assessment of tumour response [92]. The patient need only attend one examination and the overall examination time is reduced. Such imaging is not yet in widespread use but preliminary data suggest an improvement in both staging and response evaluation [93].

## I.10 Response prediction

With an increased understanding of the molecular biology of oesophageal cancer, potential molecular markers of response to neoadjuvant treatment are being identified [94]. This would allow enhanced treatment stratification as response could be predicted before initiating potentially toxic treatment and potential non-responders could be offered alternative therapies.
A number of serum and tissue molecular markers of response have been examined, predominantly using immunocytochemical staining techniques. A potential marker of chemotherapy response or resistance is the degree of expression of the target enzyme of 5-FU, thymidylate synthase (TS). Retrospective studies have indicated that TS expression may correlate with chemotherapy response in gastric cancer [95-97]. Other candidate markers of potential chemotherapy response or resistance

include the DNA excision and repair gene Excision Repair Cross Complementing 1 (ERCC1) that may be implicated in resistance to cisplatin. [95, 98, 99]

Both c-erbB2 absent expression and p53 presence in pre-treatment biopsies of oesophageal adenocarcinoma have been found to be significantly correlated with the presence of residual disease in the resected specimen [100]. Absent expression of p53 also predicted a favourable response to neoadjuvant treatment and survival.

At present, none of the tissue or serum markers of response to neoadjuvant treatment are sufficiently accurate to be used to predict response in an individual patient. One possible explanation is the multifactorial nature of carcinogenesis and the limited impact in this process caused by the presence or absence of a single molecular marker [101]. Advances in microarray technology may help to overcome these difficulties.

Further improvements in the response rates of neoadjuvant chemotherapy without increased toxicity in oesophageal cancer will only be achieved through the introduction of new chemotherapeutic agents. Drugs such as oxaliplatin, the taxanes, vinorelbine and irinotecan have all shown promising activity in the treatment of predominantly advanced or metastatic upper gastrointestinal cancers [102-110]. Several regimens have shown an encouraging therapeutic index and formal comparisons with existing cisplatin-based regimens will eventually need to be performed, which is one of the main focuses of this research project.

# Section II

## Molecular Mechanisms of Oxaliplatin and Cisplatin

### II.1 Introduction

Cisplatin-based chemotherapy constitutes a curative option for the majority of patients with advanced germ cell tumours which were previously almost uniformly fatal [111]. Cisplatin exhibits substantial efficacy and is a component of standard treatment regimens for ovarian, bladder, head and neck, oesophageal and lung cancers [112,113]. Unfortunately, despite the impressive anti-tumour activity of cisplatin in these malignancies, there are major limitations to its curative use. These include its serious side effects, relatively poor activity (intrinsic resistance) against colorectal and other common tumours and its inability to confer lasting remissions in a proportion of patients due to acquired resistance [114].

These limitations motivated the development of other platinum compounds with the double aim of circumventing cisplatin resistance and reducing its toxicity. Several thousand platinum derivatives have been synthesized and investigated. Amongst these, compounds containing a 1,2-diaminocyclohexane (DACH) carrier ligand were recognized in the early 1970s as non-cross-resistant with cisplatin [115]. DACH compounds appeared promising because they lacked the nephrotoxicity of cisplatin, were active in cell lines with acquired cisplatin resistance, and appeared to be clinically effective in tumour types intrinsically resistant to cisplatin [115].

Oxaliplatin, the most active agent among the DACH Platinum compounds when tested against cisplatin in several human cancer cell lines [116], has been found to have a wide spectrum of activity. It has proved effective in colorectal cancer (CRC) as a first-line therapy as well as in 5-fluorouracil (5-FU)-refractory tumours, and in platinum-pretreated advanced ovarian cancer [117,118]. Most importantly, in preclinical *in vitro* and *in vivo* models, oxaliplatin has been shown to be synergistic with other anticancer agents, including cisplatin, carboplatin, 5-FU and others. Several phase II clinical trials have shown efficacy of oxaliplatin combined with 5-FU for patients with previously untreated advanced gastric cancer [119-121]. The outlook for patients with advanced colorectal cancer has improved substantially with the introduction of chemotherapy agents such as irinotecan and oxaliplatin [122–124]. Median survival times for patients with metastatic tumour have almost doubled over the past 10 years as a result [125].

## II.2 History & Toxicity of Cisplatin

Cisplatin was the first platinum-containing coordination complex to be used in cancer treatment. It was first synthesised in 1844 when it was named Peyrone's chloride. In 1965 Rosenberg reported its inhibitory activity on Escherichia coli division [126] and in the early 1970's Wiltshaw and colleagues [127] carried out the first clinical studies. Cisplatin's anti-tumour activity was initially reported in chemoresistant testicular teratoma [128,129] and relapsed ovarian cancer [130]. Although this drug is known to have excellent efficacy, its increased toxicity demands its use in combination with other drugs.

Cisplatin also prolongs survival in non-small cell lung cancer (NSCLC) and has become a mainstay in the treatment of bladder, cervix, head and neck, oesophageal, small cell lung cancer (SCLC) and in several paediatric malignancies [131].

Cisplatin causes severe side effects including ototoxicity, neurotoxicity and renal toxicity. Its high emetogenic potential can be effectively controlled by administration of dopamine agonists, corticosteroids and, most effectively, serotonin antagonists, alone or in combination. [132]. However, cisplatin's ototoxicity and neurotoxicity are more difficult to control and often necessitate dose reduction or discontinuation. Ototoxicity is characterized by reversible tinnitus and irreversible hearing loss particularly affecting the high-frequency range. The severity is usually related to the cumulative dose received in subsequent therapeutic courses. Neurotoxicity is dose-limiting and characterised by peripheral neuropathy of the upper and lower limbs; paresthesias, weakness, tremors and loss of taste [132]. Renal toxicity caused by cisplatin is clinically manifested by elevation of blood urea nitrogen, serum creatinine and electrolyte disturbances.

In an attempt to overcome the side effects of cisplatin, the less toxic platinum analogue carboplatin was developed. Unfortunately carboplatin caused primary haematological toxicity [133] and necessitated the further development of new platinum derivatives and the birth of oxaliplatin.

### II.3 History & Toxicity of Oxaliplatin

Oxaliplatin is a relatively new platinum analogue that has been licensed in the European Union since 1999 and in the United States since 2002 [134]. It was first synthesised by Kidani at the University of Nagoya, Japan and developed primarily in France with the support of Roger Bellon Laboratories & Debiopharm Laboratories and now Sanofi-Synthelabo Laboratories [135]. This drug was developed as it had higher efficacy and a lower toxicity than cisplatin in vivo preclinical studies and most importantly, it had no cross-resistance with cisplatin [136-138].

Oxaliplatin has shown a broad spectrum of activity and safe toxicity profile in a wide range of human cancers. It demonstrated good results in ovarian, breast, head & neck cancer, in non-Hodgkin's lymphoma, malignant melanoma, glioblastoma and NSCLC. Its efficacy is more remarkable against cancers resistant to other platinum derivatives. The best results to date have been obtained in the treatment of colorectal cancer [139, 140]. Consequently, it has been approved for clinical use in the treatment of advanced colorectal cancer in the United States, Europe, Asia and Latin America [134]. In France it has also been used in trials of oesophageal adenocarcinomas treatment regimes [141].

Unlike cisplatin, oxaliplatin is not nephrotoxic [142]. The dose-limiting toxicity is sensory neuropathy, which has two clinical manifestations. The first is an acute dysaesthesia affecting the extremities either during infusion, shortly after the infusion or after exposure to cold temperatures. More rarely, it can cause a pharyngolaryngeal dysaesthesia [143] with difficulty in breathing or swallowing that resolves in hours and is not fatal. The second clinical manifestation is a cumulative peripheral neuropathy that usually resolves a few months following discontinuation of treatment. [144] other toxicities include nausea, vomiting, and myelosuppression. [142]

## II.4 Mechanisms of Action

Mechanisms underlying antitumor activity of cisplatin are not entirely understood despite intensive research. Structure–activity studies often employ inactive compounds such as the *trans*- isomer of cisplatin known as Transplatin [145]. Oxaliplatin shares various mechanistic properties with cisplatin. However, the precise mechanism of action of oxaliplatin is unclear and much of the knowledge is based on the extrapolation of findings for cisplatin and other DACH compounds [115].

## II.5 Chemical Structure

Cisplatin (cis-diamminedichloroplatinum II, CDDP, Fig 1) exerts antitumor activity like classical alkylating agents. The drug becomes aquated in the tissues and can then interact with macromolecules like DNA to form adducts [142 & 146].
The aquated platinum species binds preferentially to the highly nucleophilic N-7 positions of the purine bases guanine and adenine [147]. Cisplatin can also bind to RNA and cellular proteins [148].

Oxaliplatin is a third generation platinum (Pt) analogue [Fig1] that differs from cisplatin by the presence of a 1, 2 diaminocyclohexane ligand (DACH) [Fig 1], which substitutes the amine group of cisplatin in its chemical structure. Like cisplatin, oxaliplatin acts as an alkylating agent on DNA, forming platinated intrastrand cross-links between two adjacent guanine bases d(GpG) or two adjacent guanine–adenine bases d(GpA) [149, 150]. However, differences in their DNA adduct and mechanisms of DNA repair may be related to their specific activity profiles. DACH–platinum DNA adducts formed by oxaliplatin are more bulky and more hydrophobic than platinum adducts formed by cisplatin [115].

Cisplatin has two ammine groups in the cis orientation, with two chloride 'leaving' groups, a cyclobutane or a glycolate moiety. A large body of data suggests that the human metabolism consists in a double aquation of the compound, which yields an active metabolite: diaquo-diamine-platinum [135].
For oxaliplatin there is a slight variation, in that there is also a displacement of the oxalate group by H2O and Cl-, yielding monochloro-, dichloro- and diaquo-diaminocyclohexane-platinum. These metabolites seem to have the same reactivity as diamino-diaquo-platinum [135]. The diaquo-platinum species react with amine groups of proteins, RNA and DNA. The latter reaction yields platinum-DNA adducts, which appear to be associated with the clinical activity. Aquated platinum reacts preferentially with the N-7 position of guanine and adenine and produces cross-links between bases in the same strand or in opposite strands [135].

## Chemical Structures of Cisplatin & Oxaliplatin

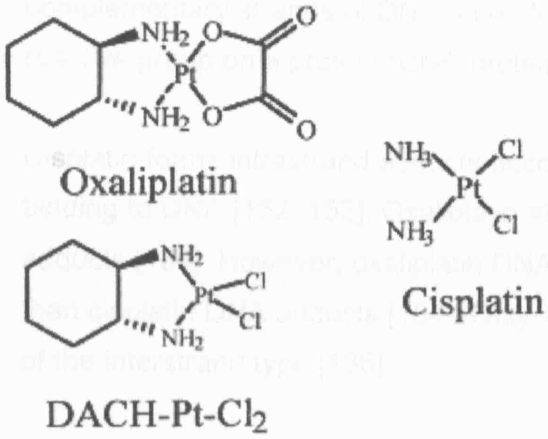


Fig 1 Structures of Oxaliplatin, cisplatin, and the reactive form of Oxaliplatin, DACH-PtCl2. [142]

## II.6 Modes of Interaction

The possible modes of interaction of cisplatin and oxaliplatin with DNA are shown in Figure 2. The formation of crosslinks requires an initial reaction to form a monoadduct. Monoadducts can go on to form other crosslinks. Crosslinks involving DNA can be on the same strand of DNA (intrastrand), between the two complementary strands of DNA (interstrand), or between a base on DNA and a reactive group on a protein (DNA-protein) [151] fig 2

Cisplatin forms intrastrand adducts accounting for well over 90% of total platinum binding to DNA [152, 153]. Oxaliplatin also forms about 90% of intrastrand platinum adducts [136]. However, oxaliplatin DNA adducts are formed 5 times more quickly than cisplatin DNA adducts [154]. Only 1% of all cisplatin and oxaliplatin adducts are of the interstrand type [136].

## Different Types of DNA Adducts

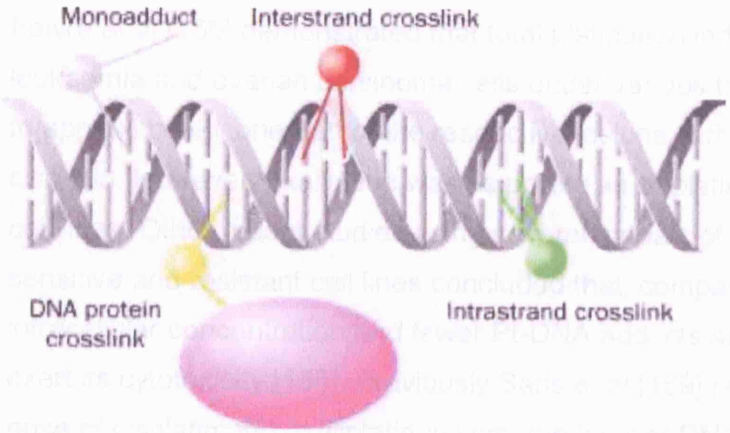


Fig 2. Possible modes of interaction of cross linking drugs with DNA [151]

## II.7 DNA Adducts

The efficacy of platinum agents against cancer cells may be related to inhibition of DNA synthesis or to saturation of the cellular capacity to repair platinum adducts of DNA [155, 156]. Trans-adducts are more easily repaired than cis-adducts. For this reason, the cis configuration of the diaquo intermediate is 30 times more toxic than the trans configuration [157].

Faivre et al [155] demonstrated that total platination induced by oxaliplatin (in human leukaemia and ovarian carcinoma cells under various treatment schemes) amounted to approximately one-half of the respective lesions in the DNA induced by equimolar cisplatin. However, oxaliplatin was as potent as cisplatin in growth inhibition of these cell lines. Other recent studies using several models of cisplatin and oxaliplatin-sensitive and resistant cell lines concluded that, compared to cisplatin, lower intracellular concentration and fewer Pt-DNA adducts are sufficient for oxaliplatin to exert its cytotoxicity [158]. Previously Saris et al [159] reported that, at an equitoxic dose of cisplatin and oxaliplatin *in vitro*, the level of DNA platination was ten times higher with cisplatin. On average, to reach the same cytotoxic effect, a 5-fold increase in cisplatin DNA adducts is required, compared to oxaliplatin DNA adducts [155, 160].

It is not apparent how different responses to DNA adducts might compensate for the reduced initial platination levels by oxaliplatin [161]. The DACH carrier ligand of oxaliplatin slowed the rate of monoadducts to diadducts conversion [162] that might increase the toxicity of oxaliplatin. Oxaliplatin DNA adducts are bulkier and induce a greater deformation of the DNA structure than cisplatin DNA adducts [163]. Consequently oxaliplatin is more effective in inhibiting DNA synthesis [164]. Some other studies have suggested that oxaliplatin DNA adducts may not be recognized by the DNA repair system in the same way as it recognized cisplatin-DNA adducts [160, 163].

Pt-DNA adducts formed by oxaliplatin, their location and their removal closely resemble the respective attributes of cisplatin-DNA adducts [158, 164]. However, oxaliplatin displays a disproportionately greater ability to induce secondary lesions in DNA that are precursors to massive apoptosis. Faivre et al [155] found that both oxaliplatin and cisplatin induce early Single Strand Break (SSB) and these early SSB probably reflect a secondary, not primary, DNA damage, as neither cisplatin nor

oxaliplatin are capable of direct cleavage of naked DNA [165]. The properties of the early oxaliplatin-induced strand breaks and their temporal progression to massive Double Strand Break (DSB) are noticeably reminiscent of apoptotic DNA fragmentation, which is generally known to progress in discrete steps that are presumably mediated by distinct sets of gradually activated nucleases [166]. The connection between the early SSB and DSB by oxaliplatin is further documented by the dependence of both these events on caspase inhibitors. The Pan-caspase inhibitor Z-VAD-fmk completely abrogates oxaliplatin induced DNA fragmentation after 24 hours treatment [167]. Other characteristic indicators of apoptotic death by oxaliplatin include the appearance of condensed chromatin that parallels massive DSB induction but clearly precedes decreases in membrane integrity. Collectively, these findings demonstrate that oxaliplatin is an apoptosis inducer as potent as cisplatin at equimolar levels [155].

## II.8 DNA Repair by Nucleotide Excision Repair System

Inhibition of DNA synthesis and repair could result from a modification of the three-dimensional structure of DNA, induced by the platinum adducts [168]. Cells with enhanced DNA repair activity are resistant to cisplatin, confirming the importance of DNA repair inhibition [169].

Nucleotide excision repair (NER) protein system is a versatile DNA repair system that removes many bulky, helix distorting DNA lesions induced by cisplatin, oxaliplatin and other DNA distorting agents [170]. NER can be divided into Global Genome Repair (GGR) for repair of lesions located anywhere in the genome and Transcription Coupled Repair (TCR) for repair of the lesions presented on the transcribed strand of active genes [57]. In TCR system, damage is detected by stalling of RNA polymerase II (RNAP-II) when it encounters a lesion at DNA [171]. Large subunit (LS) of RNAP-II (RNAP-II LS) serves as a caspase substrate [170]. Data has suggested that cells committed to apoptosis cleave RNAP-II LS. Exposure to cisplatin and oxaliplatin induces RNAP-II LS cleavage, which results in apoptosis [170, 172].

Cisplatin induces cell death by generating intrastrand DNA cross links which causes the arrest of RNAP-II [170, 173]. Cells with reduced levels of NER capability are more susceptible to cisplatin induced cell death [48]. RNAP-II stalls at DNA lesions on the transcribed strand of active genes and functions as a DNA damage sensor [170, 174]. This role of RNAP-II ensures the efficient repair of active genes and

provides a way for cells to rapidly resume the basic function of transcription after DNA damage. Cisplatin and oxaliplatin induced DNA lesions may be repaired by NER system or may induce apoptosis [170].

Increased NER activity in cisplatin resistant cell lines has been demonstrated by increased removal of DNA adducts and intrastrand cross-links [163, 175] and by the increased excision of DNA adducts in the cell extracts [176-178]. Cell culture experiments have shown that cisplatin and oxaliplatin DNA adducts were removed at the same rate [175, 177]. This data suggests that NER might not discriminate between platinum DNA adducts with different carrier ligands. Chaney et al [178] compared the ability of the human NER complex to remove cisplatin and oxaliplatin adducts in vitro and found no significant differences in the excision of DNA adducts.

### II.9 Post Replication Repair

Post-replication repair has been defined as the replication of damaged DNA without the introduction of gaps or discontinuities in DNA and /or the repair of those gaps or discontinuities following replication. Since the persistent presence of gaps or discontinuities in replicated DNA can be lethal, post replication repair is a major mechanism of DNA damage tolerance [169, 179]. In human cells, post replication repair appears to occur primarily during replication and is often referred to as replicative bypass. Many different mechanisms of DNA repair are involved in replicative bypass including NER [179].

Replicative bypass is increased in cisplatin resistant cell lines and is selective for cisplatin versus oxaliplatin adducts. That is, cisplatin resistant cell lines show increased replicative bypass of cisplatin adducts, but little or no increased replicative bypass of oxaliplatin adducts has been seen [180, 181].

### II.10 Innate and acquired chemoresistance

Resistance to cisplatin has been extensively studied and had been shown to result from several mechanisms: decreased drug transport, increased cytoplasmic detoxification by glutathione or metallothioneins, enhanced DNA repair and defects in apoptosis [182]. The mechanisms that have been shown to discriminate between cisplatin and oxaliplatin are defects in the mismatch repair complex and enhanced replication bypass mechanisms [183, 184].

The intracellular level of glutathione and other thiols has long been thought to be a determinant of cytotoxicity of cisplatin. The cellular metallothionein level has been studied extensively as a potential determinant of acquired resistance to cisplatin. Metallothioneins are small proteins rich in cysteine, and are involved in detoxification of a number of metals: cadmium, copper, lead, nickel and zinc. They may play a role as stress proteins, expressed in response to the presence of platinum complex [185]. Several enzymes are involved in the activity of glutathione including glutathione-S-transferase (GST) and glutathione synthase. The role of glutathione-S-transferase appears critical and consequently the intracellular activity of this enzyme has been proposed as a marker of resistance to cisplatin [186]. Two of the most commonly described mechanisms of resistance to cisplatin are increased glutathione concentration and reduced drug transport [187]. Sharp et al [188] determined that these mechanisms did not contribute to the acquired oxaliplatin resistance. However, other studies [158, 189] have found that elevated levels of glutathione do contribute to the oxaliplatin resistance.

Deficiencies in the apoptotic machinery are linked to some cases of cisplatin resistance [190, 191]. Cancer cells with the naturally high expression of Bcl-2 may be less susceptible to apoptosis by cisplatin [192]. Gourdier et al [193] assessed the contribution of Bcl-2 family in oxaliplatin resistance and found the modulation of Bax, Bak and Bcl-XL expression is not involved in the acquisition of oxaliplatin resistance.

Cisplatin effects are sometimes, but not always, affected by p53 status [194]. Faivre et al [155] found that p53-defective cells are not necessarily less sensitive to growth inhibition and apoptosis induction by oxaliplatin.

## II.11 Pharmacology

Little is known about how platinum agents enter the cell. An unidentified efflux pump is thought to be involved in the uptake of platinum agents [195]. Cisplatin and oxaliplatin uptake, measured over minutes to hours, increases linearly with concentration up to high external drug levels [196]. From the moment the platinum agents enter the blood stream their metabolism begins. Monoaquo and diaquo metabolites are formed at a rate that is specific to the particular platinum agent and these also react rapidly with blood components. Usually only a fraction of any drug present in the blood is available to enter cells, the fraction that is not bound to plasma proteins [143]. Oxaliplatin shows similar chemical behaviour to other platinum

derivatives and has a comparable mechanism of action. Firstly, the pro-drug oxaliplatin is activated by conversion to monochloro, dichloro and diaquo compounds by non-enzymatic hydrolysis and displacement of the oxalate group.
The kinetics of hydrolysis differs among platinum compounds, oxaliplatin being slower than cisplatin [135]. The highly reactive monochloro, dichloro and diaquo intermediates react with sulphur and amino groups in proteins, RNA and DNA. Other reactions include irreversible binding to bio-molecules such as albumin, cysteine (Cys), methionine and reduced glutathione, which are the first steps of *in vivo* biotransformation and cellular detoxification [197].

The cytotoxic activity of oxaliplatin is initiated by formation of a DNA adduct between the aquated oxaliplatin derivative and a DNA base. Initially, only monoadducts are formed but eventually oxaliplatin attaches simultaneously to two separate nucleotide bases resulting in DNA cross-links. This conversion takes more time than cisplatin but *in vitro* the two-step process is generally completed in approximately 15 minutes [198].

Substantial uptake of platinum takes place in erythrocytes where it is trapped by reactions with small intracellular bio-molecules. The maximal platinum concentration in erythrocytes is reached 3 hours after infusion. There is a slow decline in concentration with a mean half-life of 29–50 days, similar to the half-life of the erythrocytes. This indicates that, once inside, oxaliplatin is trapped in the erythrocyte and does not substantially diffuse into plasma [199].

## II.12 Elimination

Platinum complexes are essentially eliminated in the urine with very little recovered in faeces. During the first 5 days, 23-50% of cisplatin complexes are eliminated in urine [131] compared to 33-57% of oxaliplatin complexes [200, 201]. Oxaliplatin species are widely distributed among various tissue sites and free platinum is eliminated from the body mainly by renal clearance. Up to 50% of the dose is eliminated within the first day, depending on renal function, and only 2% of the dose is excreted as faeces [202].

# Section III

## Excision Repair Cross Complementing 1 (ERCC1)

### III.1 Introduction

Despite impressive governmental and industrial investment as well as intensive support from basic and translational research, the efficacy of cancer therapy remains limited. With a few notable exceptions, like testicular cancer or some haematological malignancies, currently available medicines ultimately fail to cure an advanced tumour disease. The results of adjuvant treatment are somewhat more encouraging, however the difference in relapse rates between treated and nontreated patient populations rarely exceed 10% [203, 204]. The response rates for the most of available cancer therapies fall within 20–80% for the first-line treatment, and within 5–30% for subsequent regimens. Frustratingly, a physician usually cannot predict whether a given drug will slow tumour growth in a particular patient, so the prescriptions are usually based on the statistical probability of the effect. This approach is not satisfactory for every patient.

Furthermore the major obstacles of cancer chemotherapy are the development of drug resistance and the severe side effects. Due to the modest tumour specificity of many anticancer drugs, normal tissues are also damaged. This prevents the application of sufficient high doses to eradicate less sensitive tumour cell populations. So tumours develop drug resistance that leads to treatment failure and fatal consequences for patients. [205]
Thus the consequences of administration of non-effective treatment are worse than neutral, and may include severe, even life-threatening alterations of patient's health. Therefore, the question arises as to which particular cytostatic agent and which combination of substances is most suited for an individual tumour. While the statistical probability of therapeutic success is well known for larger groups of patients from clinical therapy trials, it is, however, not possible to predict how an individual tumour will respond to chemotherapy. Although clinicopathological prognostic factors such as tumour size, lymph node, and distance metastases are valuable for the determination of prognosis of larger cohorts, they are less helpful for the development of personalized therapy options. Novel strategies to broaden the narrow therapeutic range by separating the effective dose and toxic dose would be of great benefit for the improvement of cancer chemotherapy [206]

The last but not least concern is the economical challenge. Many of recently approved therapy regimens are extremely costly; therefore even the wealthiest countries in the world are forced to reconsider cancer treatment related expenses. Personalized approach to antitumor therapy appears to be favourable from a budgetary perspective as well since it may reduce a non-effective use of expensive compounds [206-207].

While assessing the individual probability of benefit from an anticancer drug, oncologists currently rely only on a few phenotypic characteristics, such as tumour histology and grade, patient's performance status, etc. It is believed, that identification of DNA repair genes as markers of response to platinating or alkylating agents like ERCC1 which is a key enzyme in the NER repair pathway, may be a step forward in designing the personalized chemotherapy [208]

## III.2 Nucleotide Excision Repair System

The repair of platinum–DNA lesions is believed to occur primarily by the process of NER. Around 30 proteins participate in NER repair process; ERCC1 is one of them. It is a 15-kb repair gene located on human chromosome 19. DNA damage is thought to be recognized by protein XPA in association with the heterotrimeric replication protein RPA. The XPA–RPA complex then is believed to recruit the basal transcription factor TFIIH, a multisubunit protein that also plays a role in transcription, to the site of damage. Two subunits of TFIIH, XPB and XPD, have helicase activities that are believed to function in opening up the DNA around adduct, thus allowing structure-specific nucleases to incise the DNA. The ERCC1–XPF heterodimer is responsible for cutting the strand on the 5′ side of the damage, and the XPG protein incises on the 3′ side [151, 209]. Finally, the resulting gap filled in and ligated using the remaining undamaged strand as the template by DNA polymerases in the presence of replication factors [151] [Fig 3]

Cisplatin induces monoadducts, intrastrand and interstrand cross-links in DNA [210, 211]. Cells expressing ERCC1, repaired interstrand cross-links more effectively than ERCC1 deficient cells; whereas for intrastrand cross-links a similar effect of ERCC1 was not found [212]. Koberle and colleagues established that human testicular cancer cell lines are defective in NER because they have very low amounts of the XPA and ERCC1-XPF proteins [213] thus they are sensitive to cisplatin. However defects of different proteins involved in NER do not seem to contribute equally to a

hypersensitive cellular phenotype. For instance, ERCC1 and ERCC4 defective cells are more sensitive than ERCC2 and ERCC3 mutations to various anticancer agents, including cisplatin [214].

In addition to ERCC1, cisplatin sensitivity is also highly dependent on the DNA repair protein XPA. XPA is a critical player in DNA damage recognition and has identified as critical to cell's ability to respond to cisplatin insult. Extremely low levels of XPA and to a lesser degree ERCC1 were found responsible for the pronounced cisplatin sensitivity in Non Seminoma cell line. Elevated levels of XPA were found in cisplatin-resistant tumours [215]. Not all investigators however, have found this correlation to hold [216-219]

## Key Steps in DNA Repair by NER System

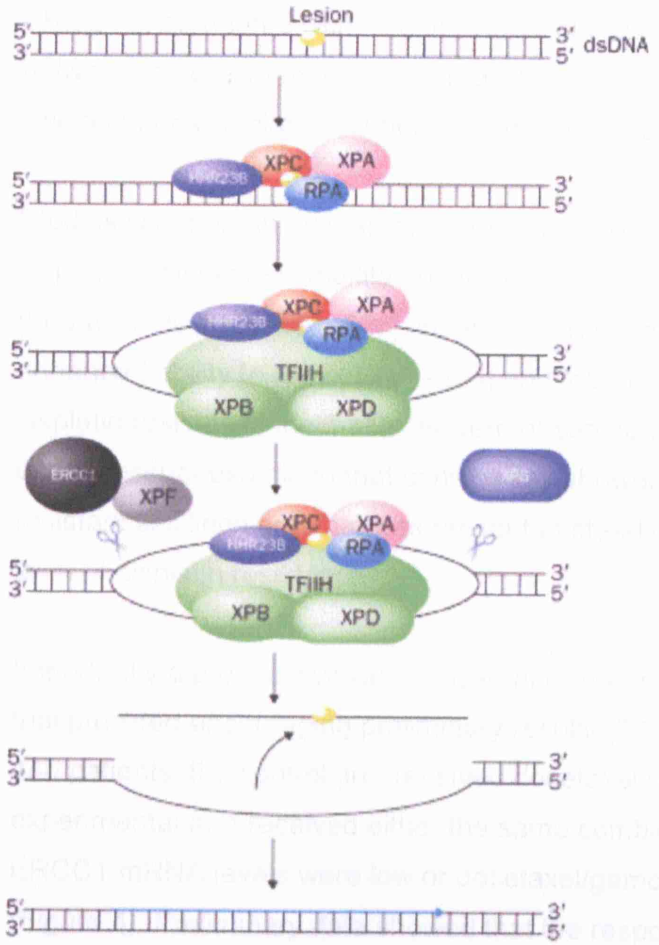


Fig 3 DNA damage recognition by XPC-hHR23B and/or XPA/RPA (Replication Protein A) [6 & 217] a large complex, which shares identity with the RNA polymerase II, transcription factor TFIIH, is recruited. Critically, this complex contains helicases (XPB and XPD), which open a DNA segment approximately 30-base-long around the damage in an ATP-dependent fashion [218]. This open intermediate is stabilized by RPA and XPA. The DNA strand that contains the damaged base is excised by the two NER endonucleases, XPG and XPF/ERCC1. XPG cleaves the damaged DNA strand 3′ from the lesion, and XPF/ERCC1 cleaves the damaged strand 5′ from the DNA lesion. Finally, the resulting gap filled in and ligated using the remaining undamaged strand as the template by DNA polymerases in the presence of replication factors [151]

## III.3 Role of ERCC1 in Chemotherapy & Clinical Application

Several independent investigations demonstrated that high intratumoral level of ERCC1 is correlated with low efficacy of platinum-based therapy. An association between increased expression of ERCC1 and resistance of ovarian cancer to cisplatin or carboplatin has been demonstrated as early as in 1994 [215].

Studies with human ovarian cancer cell lines (A2780/C) [220] have indicated that cisplatin resistance is multifactorial, consisting of mechanisms such as decreased drug accumulation [221–223], increased drug inactivation [224 –228], and an enhanced ability to repair [229 –234] and tolerate [235] DNA damage. All of these cisplatin resistance mechanisms were observed in these cell lines. ERCC1 was the only transcript examined that consistently showed a significant increase in all of the resistant cell lines and that was present at steady-state levels that correlated with the level of cisplatin resistance.

Importantly a prospective randomised and customized lung cancer chemotherapy trial provided encouraging preliminary results [236, 237]. In this ERCC1 mRNA trial of 440 patients, the control arm received docetaxel plus cisplatin; patients in the experimental arm received either the same combination of docetaxel/cisplatin if their ERCC1 mRNA levels were low or docetaxel/gemcitabine if their levels were high (Figure 4). Preliminary data showed that the response rate for patients with low ERCC1 levels was significantly higher than for patients in the control arm [238]

## ERCC1 randomised trial plan

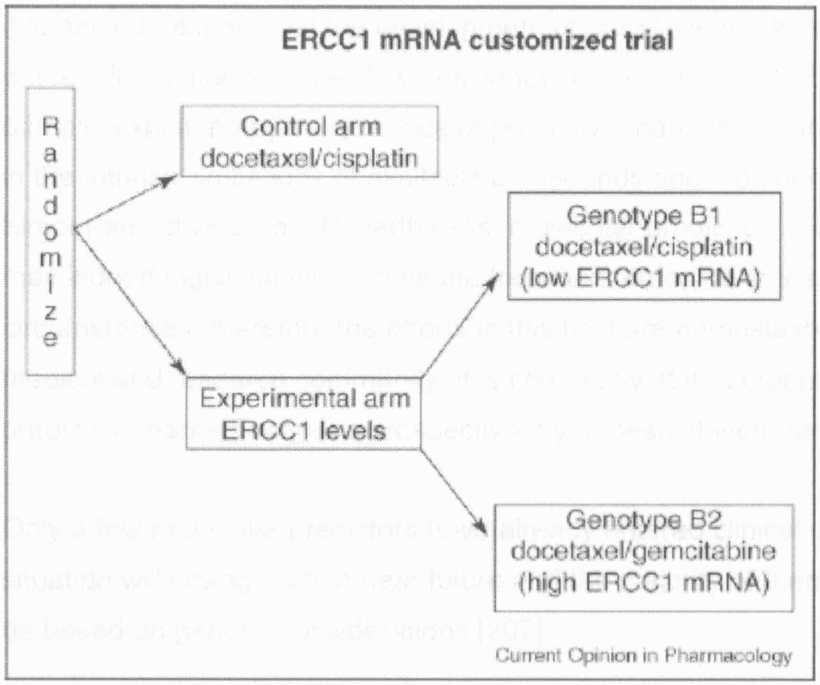


Fig 4 ERCC1 mRNA-based randomised trial of customized chemotherapy [237]

In Lord et al [239] study 56 NSCLC patients treated with gemcitabine/cisplatin demonstrated that high levels of the gene transcript (ERCC1>4.5) were associated with reduced likelihood of response rate (RR )36% compared with 58% in patients with ERCC1 <4.5 [239]. ERCC1 was over expressed in approximately half of NSCLC patients, and preliminary clinical data indicated that it is linked to significantly shorter survival. In contrast, in tumours with low ERCC1 expression, median survival exceeds 1 year [240]

In Metzger et al [95] demonstrated for the first time that intratumoral levels of ERCC1 influence the outcome of gastric cancer patients treated with cisplatin/FU, however this study gave no conclusive results on whether ERCC1 levels could be an independent predictive marker for cisplatin benefit. Along the same lines, ERCC1 levels have been correlated with oxaliplatin resistance in colorectal cancer patients. Median survival for patients with ERCC1 expression <4.9 was 10 months, while for patients with ERCC1 expression >4.9, it was 1.9 months [219]. These findings indicate that intratumoral ERCC1 may act as an independent predictive marker for oxaliplatin combination chemotherapy.

### III.4 Perspectives

It is naïve to expect that the development of predictive markers for tumour therapy will result in a cancer cure. The deficiency of current treatment approaches is only partially explained by the absence of predictive markers, but the main drawback lies in the intrinsic limitations of available compounds and the shortage of novel, truly tumour-selective drugs. Nevertheless, molecular prediction of tumour responsiveness may indeed significantly improve the treatment outcome at least in some circumstances, therefore the efforts in this field are enthusiastically supported by medical and research community. It is commonly stated that proper validation of predictive markers requires prospective, hypothesis-driven, randomised clinical trial.

Only a few molecular predictors have already entered clinical use. It is hoped that the situation will change within near future and the majority of therapeutic decisions will be based on genetic considerations [207]

# Section IV

## Bcl-2 family

### IV.1 Introduction

B-cell lymphoma 2 (Bcl-2) proteins were discovered as oncoprotein that supports neoplastic growth, not by stimulating cellular proliferation, but rather by blocking cell death [241]. In recent years, a number of proteins functionally and structurally related to Bcl-2 have been discovered [242]. Bcl-2 family proteins consist of at least 20 members in mammals. Members of this family are subdivided into, anti-apoptotic members, such as Bcl-2, Bcl-$X_L$ & Bcl-w and pro-apoptotic members, such as Bax, Bak, and Bok [243] Fig 5

## The Bcl-2 Family and Their Protein Domain Organisation

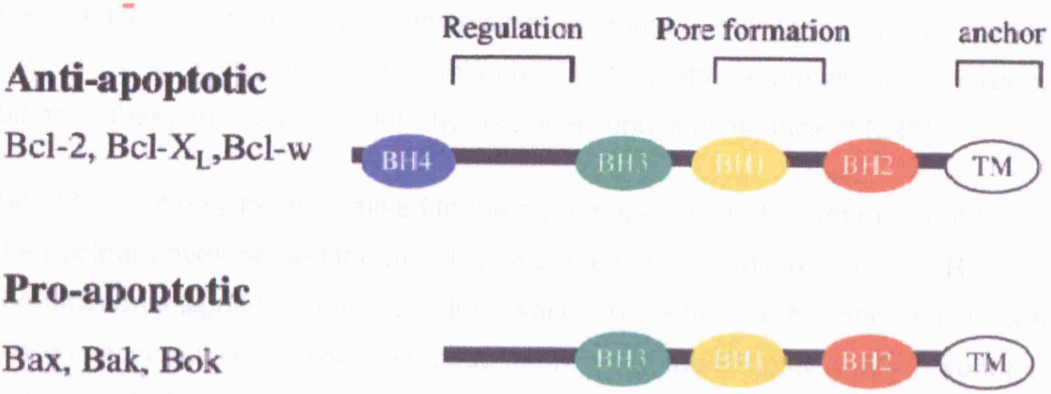


**Fig 5** The Bcl-2 family and protein domain organisation. Abbreviations: BH1-4, Bcl-2 homology domains; TM, transmembrane domain [243]

The precise biochemical function of the Bcl-2 family is far from being understood. Nevertheless, in recent years their impact on the mitochondrial membrane has become the central focus of scientific interest.

## IV.2 Bcl-2 Functions

Bcl-2 inhibits apoptosis by preservation of the mitochondrial membrane integrity [244]. Bcl-2 prevents Bax/Bak oligomerisation somehow that would otherwise lead to the release of several apoptogenic molecules from the mitochondria. Like Bax and Bak, Bcl-2 is inserted in the outer mitochondrial membrane but whether it directly binds to these molecules under physiological conditions is unclear [245].

Bcl-2 has not only been localised to the outer mitochondrial membrane but also to the nuclear envelope and the membrane of the endoplasmic reticulum (ER). While the functional significance of Bcl-2 localisation in the nuclear membrane is uncertain, there is accumulating evidence that components of the ER play a role in apoptosis induction [246]. ER-associated Bcl-2 is able to protect cells from various types of apoptosis and it may do so by scavenging pro-apoptotic Bcl-2 family members, thereby preventing, for example, their translocation to the mitochondria [247].

## IV.3 Bax Functions

Bax is a cytosolic protein that upon apoptotic stimulus changes its conformation and translocates to the mitochondria [244]. Stress signals provoke the C terminus to flip out and mediate Bax integration into the mitochondria and its subsequent oligomerisation which releases apoptogenic factors like cytochrome c from the mitochondria and sets the stage for the apoptosomal amplification loop generating Casp-9/Casp-3 activity [245].

## IV.4 Bcl-2 Family and Cisplatin

Dunkern and colleagues [248] compared the NER deficient Chinese hamster ovary (CHO) mutant fibroblasts 27-1 and 43-3B defective in ERCC3 and ERCC1, respectively, as to their sensitivity to cisplatin (measured by reproductive cell death), and specifically addressed the question of whether hypersensitivity is caused by the induction of apoptosis, or necrosis, or both. Furthermore, they analysed apoptotic signalling involved in the execution of cell death in these NER defective cells. Results demonstrated that ERCC1 and ERCC3 CHO mutants display a high level of cisplatin-

induced apoptosis, Bcl-2 decline and caspase activation, indicating that non-repaired cisplatin-induced DNA lesions act as trigger of the mitochondrial apoptotic pathway.

The most prominent proteins involved in the regulation of the mitochondrial damage pathway are Bcl-2 and Bax. Western blot analysis of cisplatin-treated cells revealed a significant decrease in anti-apoptotic Bcl-2 protein in both NER deficient cell lines. The decline was more pronounced in 43-3B than in 27-1 cells in accordance with the higher sensitivity of the former cell type. Bcl-2 decline was first observed 24 h after treatment and therefore preceded the appearance of apoptotic cells. The pro-apoptotic Bax protein was not affected in its expression level within 72 h after treatment in 27-1 cells. There was however, some decrease of Bax in 43-3B cells (up to 40% of the control level) 72 h after treatment.

Cisplatin-induced apoptosis in CHO cells appears to be regulated by changes in the Bax/Bcl-2 expression ratio, as a decline of Bcl-2 protein level was observed on cisplatin treatment. Bcl-2 decline was more pronounced in 43-3B than in 27-1 cells. The most refractory were the wild-type and the ERCC1 complemented 43-3B cells, indicating that non-repaired DNA damage is responsible for this effect. Bcl-2 decline thus appears to be a hallmark of DNA damage-induced apoptosis in fibroblasts. Regarding the specific lesion induced by cisplatin triggering this response, it is important to note that ERCC1 deficiency affected the repair of DNA interstrand but not of intrastrand cross-links [249]. Considering the high sensitivity of ERCC1 mutants, it is reasonable to suppose that DNA interstrand cross-links are the major cytotoxic lesions induced by cisplatin. It appears that non-repaired interstrand cross-links or DNA breaks arising from them [250] trigger the apoptotic pathway via Bcl-2 decline and caspase-9 and -3 activation}

The involvement of Bcl-2 in cisplatin resistance however is supported by transfection experiments of Bcl-2 in human bladder cells not expressing Bcl-2, leading to cisplatin resistance [251]. Furthermore, cisplatin resistant human epidermal carcinoma cells showed an increase in the amount of Bcl-2 [252].

### IV.5 Oncogenic Functions of Bcl-2 Family

According to the results from cell culture and animal models, down-regulation or inactivation of pro-apoptotic Bax-like death factors is observed in several human cancers. Decreased Bax levels in tumours are not surprising, given the fact that Bax

is a transcriptional target of the tumour suppressor p53 that is mutated in the majority of human cancers [253]. Impaired Bax expression has been reported in breast cancer [254], hepatocellular carcinomas [255] and in a number of other tumour types. Deregulation of Bcl-2 family members has been tightly linked to tumorigenesis [256]. All anti-apoptotic Bcl-2 homologues seem to function as oncoproteins, and pro-apoptotic BH3-and Bax-like proteins can act as tumour suppressors.

### IV.6 Bcl-2 Family in Chemotherapy

The fact that defects in apoptosis can promote drug resistance strongly suggests a causal relationship between apoptosis and drug-induced cytotoxicity [257]. In this context, up-regulation of the anti-apoptotic and down-regulation of the pro-apoptotic Bcl-2 family members in tumours have been associated with their decreased susceptibility to the action of chemotherapeutics. Significant abrogation of Bcl-2/Bcl-XL expression as well as reinforcement of Bax expression not only cause tumour regression but also render them more sensitive to apoptosis-inducing treatment [258].

## Section V
## Caspase 3

### V.1 Caspases

Caspases are a group of cysteine proteases recognising substrate specificity for aspartic acid at the P1 C-terminal site. There are 14 mammalian caspases ranging in size from 32 to 55 kDa [259]. All except caspase-11 to caspase-13 are found in humans. Caspases undergo activation by autoproteolytic processing, oligomerization, and heterocleavage by other caspases or granzyme B [260]. Long proarm caspases (e.g., caspase-2, caspase-8, caspase-9, and caspase-10) are considered initiator caspases and are the first line of activation after a cell death initiation signal triggers cellular apoptosis. Short proarm caspases (caspase-3, caspase-6, and caspase-7) are mostly activated through the action of initiator caspases and are named effecter caspases because their activation is believed to result in irreversible cell damage leading to cell death. A number of caspases (e.g., caspase-1, caspase-4, caspase-5, caspase-11, and caspase-14) are implicated in the activation of pro inflammatory cytokines and their activation may not necessarily lead to apoptosis [261]. Evidence suggests that caspase activation does not always result in cell death and that caspases may have physiological functions other than cell death [262].

## V.2 Cisplatin Stimulated Apoptosis

Dunkern and colleagues [248] measured caspase activation in NER deficient cells as a response to cisplatin for the initiator caspases-9 and -8 and the executioner caspase-3 using cell extracts of 43-3B and ERCC1-complemented 43-3B cells (NER deficient Chinese hamster ovary (CHO) mutant fibroblasts). Treatment of 43-3B/ERCC1 cells with cisplatin did not cause caspase activation, however activation of all three caspases was observed in 43-3B cells upon treatment with cisplatin. The activation of caspase-3 was most pronounced (7.5-fold above the control level) as compared to caspase-9 and -8 (enhanced by factor of 2.5).

Cisplatin-induced apoptosis in the NER deficient mutants appears to be controlled by the mitochondrial apoptotic pathway, which obviously becomes activated by specific non-repaired DNA damage. It is unlikely that the direct activation of membrane bound death receptors is mainly involved in cisplatin-induced cell killing, because the frequencies of apoptosis and necrosis are related to the DNA repair deficiency. Cisplatin-induced apoptosis in CHO cells appears to be regulated by changes in the Bax/Bcl-2 expression ratio as a decline of Bcl-2 protein level was observed upon cisplatin treatment. Bcl-2 decline was more pronounced in 43-3B than other cells. The most refractory were the wild-type and the ERCC1 complemented 43-3B cells, indicating that non-repaired DNA damage is responsible for this effect [248].

Bax is involved in releasing of cytochrome C from mitochondria [263]. Cytochrome C, Apaf-1 and ATP assemble with pro-caspase-9, creating the apoptosome complex [264]. This complex generates the active caspase-9 which in turn activates the executioner caspase-3. Caspase-9 may also activate caspase-8. In fact, they observed all these caspases to be activated upon cisplatin treatment in 43-3B cells, but not in the same cell type in which the repair defect was complemented by transfection with ERCC1. This indicates that caspases were triggered by non-repaired DNA damage, very likely via down-regulation of Bcl-2.

A sensor of DNA damage is p53 which is known to control the expression of several apoptosis-regulating proteins like Bax [265] and Bcl-2 [266]. However, CHO cells are mutated for p53 [267]. Therefore, apoptosis induced by non-repaired cisplatin adducts in this cell system is independent from functional p53.

CHO cells are unable to undergo tumour necrosis factor (TNF) stimulated receptor-mediated apoptosis thus it seems that the death receptor signalling pathway is not functional in these cells. As activation of caspase-8 was detected in the NER defective CHO mutants, it was supposed that it was a down-stream event of caspase-3 activity rather than of death receptor triggered activation, which was also concluded by others using other experimental systems [264, 268–270].

## Aims and Purposes of this Research

With an increased understanding of the molecular biology of oesophageal cancer, potential molecular markers of response to neoadjuvant treatment are being identified. This would allow enhanced treatment stratification as response could be predicted before initiating potentially toxic treatment and potential non-responders could be offered alternative therapies

This thesis describes a series of in-vitro experiments that examine the effects of cisplatin and oxaliplatin on ERCC1 and XPA genes involved in DNA repair and apoptosis in oesophageal adenocarcinoma (OE33) and squamous cell carcinoma (OE21) cell lines. Several independent investigations demonstrated that high intratumoral level of ERCC1 is correlated with low efficacy of platinum-based chemotherapy. Thus experiments in this project were designed to see if there was in-vitro evidence to support a clinical study at a later date.

In UK, cisplatin combinations are used for oesophageal cancers. Oxaliplatin, a derivative of cisplatin has shown good results in colorectal cancer. In this work sensitivities of cisplatin and oxaliplatin in the OE 33 and OE 21 cells were also compared in order to investigate whether oxaliplatin is having the same good results in oesophageal cancer cells as it has shown in colorectal cancers. These sensitivities were compared using AlamarBlue™ as cell viability studies. Gene expression of ERCC1, XPA, Bcl-2, Bax and Caspase 3 were analysed using RT-PCR techniques.

# Chapter 2
## Sensitivities of OE33 and OE21

## 2.1 Cell Lines Description

European Collection of Animal Cell Cultures (ECACC) has described the following description of OE33 and OE21 cell lines.

| | |
|---|---|
| **ECACC No.** | **96070808** |
| **Cell Line Name** | **OE33** |
| **Keywords** | Human Caucasian oesophageal carcinoma |
| **Cell Line Description** | The cell line OE33, also known as JROECL33, was established from the adenocarcinoma of the lower oesophagus (Barrett's metaplasia) of a 73 year old female patient. The tumour was identified as pathological stage IIA (UICC) and showed poor differentiation. OE33 express HLA-A, -B and -C antigens (MHC class I) and ICAM-1 constitutively. Expression of HLA-DR (MHC class II) can be induced by treatment with interferon-gamma. The cells express epithelial cytokeratins and are tumourigenic in nude mice. |
| **Species** | Human |
| **Tissue** | Oesophagus |
| **Morphology** | Epithelial |
| **Passage Number** | 7 |
| **Sub Culture Routine** | Split sub-confluent cultures (70-80%) 1:8 i.e. seeding at 1x10,000 cells/cm² using 0.25% trypsin or trypsin/EDTA; 5% CO2; 37°C. Initially these cells grow slowly and can take up to 7 days until ready for the next split, 50% media changes will be necessary every 2-3 days (i.e. replacing half the old medium with fresh). |
| **Culture Medium** | RPMI 1640 + 2mM Glutamine + 10% Foetal Bovine Serum (FBS). |
| **Karyotype** | Aneuploid |
| **Receptors** | MHC class I, ICAM-1 |
| **Depositor** | Drs J C Rockett/A Morriss, Department of Biological Sciences, University of Warwick and Dr S J Darnton, Birmingham Heartlands Hospital |
| **Originator** | Yes |
| **Country** | UK |
| **References** | Br J Cancer 1997;75:258 |

| | |
|---|---|
| **ECACC No.** | **96062201** |
| **Cell Line Name** | **OE21** |
| **Keywords** | Human Caucasian oesophageal squamous cell carcinoma |
| **Cell Line Description** | OE21, also known as JROECL21, was established in 1993 from a squamous carcinoma of mid oesophagus of a 74 year-old male patient. The tumour was identified as pathological stage IIA(UICC) and showed moderate differentiation. HLA-A,-B and -C (MHC class I) are expressed constitutively, expression of ICAM-1 can be induced by treatment with interferon-gamma. OE21 cells express epithelial cytokeratins and are tumourigenic in nude mice. |
| **Species** | Human |
| **Tissue** | Oesophagus |
| **Morphology** | Epithelial |
| **Passage Number** | +3 |
| **Sub Culture Routine** | Split sub-confluent cultures (70-80%) 1:8 i.e. seeding at 1x10,000 cells/cm² using 0.25% trypsin or trypsin/EDTA; 5% CO2; 37°C. |
| **Culture Medium** | RPMI 1640 + 2mM Glutamine + 10% Foetal Bovine Serum (FBS). |
| **Karyotype** | Aneuploid |
| **Receptors** | MHC class I |
| **Depositor** | Dr J C Rockett, Dr AG Morris, Department of Biological Sciences, University of Warwick and Dr S J Darnton, Birmingham Heartlands Hospital |
| **Originator** | Yes |
| **Country** | UK |
| **References** | Br J Cancer 1997;75:258 |

## 2.2 Characteristics of OE33 and OE21 cells

Working with cell lines in monolayer culture implies a lot of difficulties regarding the comparability of the behaviour to the complex in vivo situation including growth and resistance mechanisms. Three-dimensional multicellular spheroids (MCS) mimic the growth characteristics of solid tumours and to develop gradients in nutriens like oxygen and degradation products with increasing size. Cells cultivated as MCS are highly differentiated than in monolayer culture and have a similar extracellular matrix system. The model simulates solid micrometastases and microregions between blood vessels and enables experiments concerning the microenvironment and formation of subpopulations. Ling and colleagues [271] studied such a three-dimensional model for OE21 and OE33. They report the successful establishment of MCS derived from established cell cultures of oesophageal carcinomas. Cell morphology was completely different depending on their tumour origin. The MCS displayed a higher differented morphology compared to the respective monolayer

cultures, OE21: bulky spheroids OE33: small sheroids. Lethal cells and necrotic areas were predominantly detectable in OE21 sheroids. Reactive oxygen species were intensively detected in adenocarcinoma sheroids compared to squamous cell cancer suggesting similarity to the in vivo situation [271]

## 2.3 Manipulation of Cell Cultures

The ability to maintain and manipulate cells in culture is a valuable tool for life science researchers. Cell culture models permit the study of a single cell type. Cell culture models also permit measurement of cell responses to conditions which are more tightly regulated than the complex environment presented by an entire organism. Perhaps the most fundamental measurements which can be made with cells in culture are viability and proliferation [272]

Determination of cell viability and proliferation enables researchers to:
• optimise cell culture conditions,
• quantitate the activity of cell growth factors including cytokines,
• facilitate the discovery of new therapeutic agents such as antibiotics and anti-cancer agents,
• assess the efficacy of therapeutic agents such as antibiotics and anti-cancer agents,
• assess cell mediated toxicity,
• quantitate apoptosis.

## 2.4 Available Methods of Assessing Cell Viability and Proliferation

Methods of assessing cell viability include measurement of Trypan Blue exclusion or propidium iodide exclusion, and measurement of Neutral Red inclusion, CFDA inclusion, or crystal violet inclusion. Cell death (for example, by cell mediated cytotoxicity) is measured by the quantitation of $^{51}Cr$ (chromium) or Europium Titriplex V release. AlamarBlue ™ is used to assess viability in response to toxic agents.

Methods of measuring cell proliferation include the detection of proliferation associated antigens by immunohistochemistry, quantitation of DNA synthesis by measuring tritiated thymidine (3H-thymidine) or bromodeoxyuridine (BrdU) uptake or by measuring changes in total DNA content with DNA specific dyes such as Hoechst 33258. Proliferation may also be measured by quantitation of the reduction of the

intracellular environment by tetrazolium salt reduction or alamarBlue™ reduction [272]

## 2.5 AlamarBlue™ in comparison with other agents

AlamarBlue™ is a novel dye for cell viability studies. It is a safe (non-toxic, non-carcinogenic) aqueous dye that gives superior results when compared to MTT, XTT or neutral red in cell proliferation assays.

.

Viability and proliferation measurements obtained with AlamarBlue ™ compare favourably with measurements obtained by other methods. Specifically, AlamarBlue ™ reduction, when measured spectrophotometrically, produces linear results over a similar range of concentrations of cells as MTT (3-(4,5-dimethyethiazol-2-yl)-2,5-diphenyltetrazolium bromide), and XTT (sodium 3'-[1-phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrateand). AlamarBlue™ permits monitoring over a broader range of cell densities than MTT and XTT. When reduction is monitored by fluorescence [273] AlamarBlue ™ is substantially less expensive to use than MTS.

Results obtained in the measurement of cell mediated cytotoxicity comparing the use of AlamarBlue ™ with $^{51}Cr$ release assays indicated that the AlamarBlue ™ method is as specific as determination of $^{51}Cr$ release but that the AlamarBlue ™ method is more sensitive, requiring fewer effecter cells [274] and is, of course, non-radioactive.

In a toxicology study, the IC50 for daunorubicin is similar when measured with XTT and AlamarBlue ™ [275]. Because AlamarBlue ™ is efficiently reduced; it has the advantage over XTT in that it does not require treatment with PMS (phenozine methosulfate). In the determination of a minimal number of proliferating cells, measurement of AlamarBlue ™ reduction is at least as sensitive as measurement of tritiated thymidine incorporation [276].

## 2.6 AlamarBlue ™ Mechanism of Action

The AlamarBlue™ assay incorporates a fluorometric /colorimetric growth indicator based on detection of metabolic activity. The specific (fluorometric /colorimetric) REDOX indicator incorporated into AlamarBlue exhibits both fluorescence and

colorimetric change in the appropriate oxidation-reduction range relating to cellular metabolic activity.
All cells being tested grow innate metabolic activity results in a chemical reduction of AlamarBlue. Continued growth maintains a reduction environment while inhibition of growth maintained an oxidized environment. Reduction related to growth causes the REDOX indictor to change from oxidized (non-fluorescent, blue) form to reduced (fluorescent, red) form [277]

A common measure of cytotoxicity is LD50 determination.
To determine LD50 with AlamarBlue ™, cell growth in the presence and absence of the compound under consideration is compared. Measurements are made either with the spectrophotometer at two wavelengths or by fluorescence. Data obtained from this comparison are plotted as percent inhibition on the y axis and log of concentration of the compound under consideration on the x axis. The LD50 is determined from this curve corresponding to the concentration at which 50% inhibition is observed.

## 2.7 Determination of Cisplatin and Oxaliplatin LD50

Lethal dose 50 (LD50) is the quantity of a drug which would be needed to kill 50% of any particular cell line. The lesser the amount of a drug needed to induce 50% cell death the more cytotoxic it would be to that particular cell line or vice versa [277]. LD50s for cisplatin and oxaliplatin in OE 33 and OE 21 cell lines were determined using AlamarBlue™ assay.

## 2.8 Materials and Methods

The OE33 & OE21 cells were grown in supplemented media 1640 RPMI at 37°C in an atmosphere containing 5% $CO_2$. Cells were passaged once a week using 0.25% trypsin/EDTA. Doubling time for OE 33 was 26 hours and for OE 21 cells 21 hours. Cells were platted in 24 wells plate with cell density of $1x10^5$ per well in serum positive media. Plates were incubated for 4 hours. Nine plates for each drug were treated with decreasing concentration of cisplatin and oxaliplatin from 1millimolar to 50ηmolar in serum free media except first row of the wells which was left untreated for control. Treatment time was 24 hours. 1ml of 10% AlamarBlue™ solution was added in each well and left over for 24 hours. Cell viability was assessed by using two different programmes.

Multiskan Lab system was used for colorimetric plate readings and assessing the AlamarBlue™ absorbance monitored at 570nm and 600nm wavelength and Thermo Lab system was used for fluorometric plate readings monitored at 530-560nm excitation wavelength and 590nm emission wavelength using Ascent Software version 2.6 for assessing AlamarBlue™ excitation and emission.

## 2.9 Statistics

Cytotoxicity values obtained, either from Multiskan Lab system, (which reads colorimetric absorbance of alamarBlue assay) or fromThermo Lab system (that takes fluorometric plate readings with Ascent Software version 2.6) are inserted into Excel®. First, an average was calculated for all values separately including control wells. Then each average was divided by the control average value individually. Each value was multiplied with 100. it has given the final reading for each well separately, as each value represent a well with a specific drug concentration. These values were inserted against specific drug concentration in Graph Pad Prism which calculated the LD 50.

All the cytotoxicity assay data were analysed by using Prism Graph Pad Version 4 and SPSS. Log molar concentrations of each drug were plotted against a common average cell viability value in Graph Pad Prism 4 programme to determine the lethal dose effective Concentration (EC50) for cisplatin and oxaliplatin, which is approximately equivalent to LD50. For detailed p value calculations please see the appendix three.

## 2.10 Results for OE 33

EC50 dose of cisplatin was 30μM (approx) (figure 6) and for oxaliplatin 75μM (approx) (figure 7). Prism programme has taken the maximum and minimum values to calculate the EC 50 values that represent the dose required to bring the cell viability to the mid point between the maximum and minimum, which approximately represent the LD50.

In case of cisplatin it takes 97% as maximum and 57% as minimum, the mid point therefore is 77% (which in other words represent 50% mark) at this level of cell viability percentage the log of cisplatin is -4.521M, which is when antiloged gives an EC 50 of $3.101 \times 10^{-5} = 30\mu M$

In case of oxaliplatin it takes 93% as maximum and 50% as minimum, the mid point therefore is 74% (which in other words represent 50% mark) at this level of cell

viability percentage the log of oxaliplatin is -4.097M, which is when antiloged it gives an EC50 of $7.91x10^{-5}$ = 79μM. p values were calculated by comparing the EC50s between cisplatin and oxaliplatin. The p value for OE 33 was 0.009, which is quite significant. These values suggested that cisplatin might be more effective than oxaliplatin in inducing apoptosis in oesophageal adenocarcinoma cells OE 33.

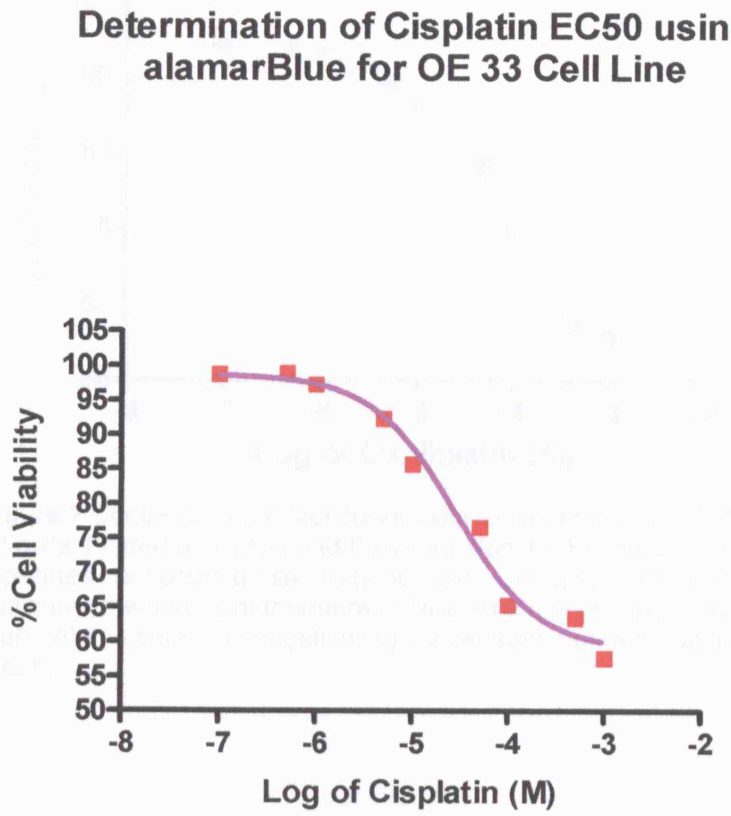


**Figure 6** Concentration effect curve used to determine the EC50 value for cisplatin in OE 33 cells. Plotted on Graph pad Prism Version 4.0, each data set represents the mean of 6 replicates conducted on separate occasions. Prism here takes 97% as maximum and 57% as minimum; the mid point therefore is 77%, which in other words represent 50% of cell viability mark. At this point, the negative log concentration of cisplatin is -4.521M as shown in the graph

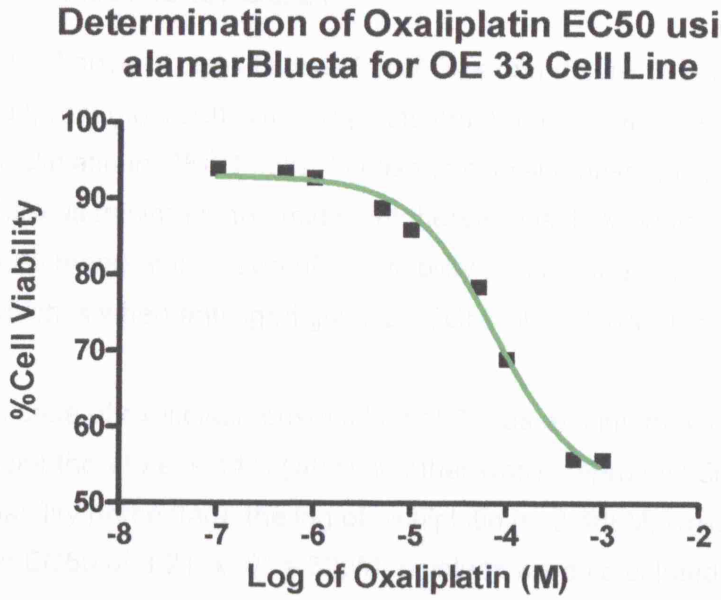


**Figure 7** Concentration effect curve used to determine the EC 50 value for oxaliplatin in OE 33 cells. Plotted on Graph pad Prism Version 4.0 Each data set represents the mean of 6 replicates conducted on separate occasions. Prism here takes 93% as maximum and 50% as minimum; the mid point therefore is 74%, which in other words represent 50% of cell viability mark. At this point, the negative log concentration of oxaliplatin is -4.097 as shown in the graph.

## 2.11 Results for OE 21

EC50 dose of cisplatin was 25μM (approx) (figure 8) and for oxaliplatin 35μM (approx) (figure 9). This suggests that both cisplatin is slightly more cytotoxic than oxaliplatin in OE 21 cells. In case of cisplatin prism takes 100% as maximum and 10% as minimum, the mid point therefore is 45% (which in other words represent 50% mark) at this level of cell viability percentage the log of cisplatin is -4.566M, which is when antiloged gives an EC50 of $2.7157 \times 10^{-5}$ = 27μM

In case of oxaliplatin prism takes 100% as maximum and 12% as minimum, the mid point therefore is 44% (which in other words represent 50% mark) at this level of cell viability percentage the log of oxaliplatin is -3.488M, which is when antiloged it gives an EC50 of $3.211 \times 10^{-5}$ = 32μM. p values were calculated by comparing the EC50s between cisplatin and oxaliplatin. The p value for OE 21 was 0.14009, which is not significant.

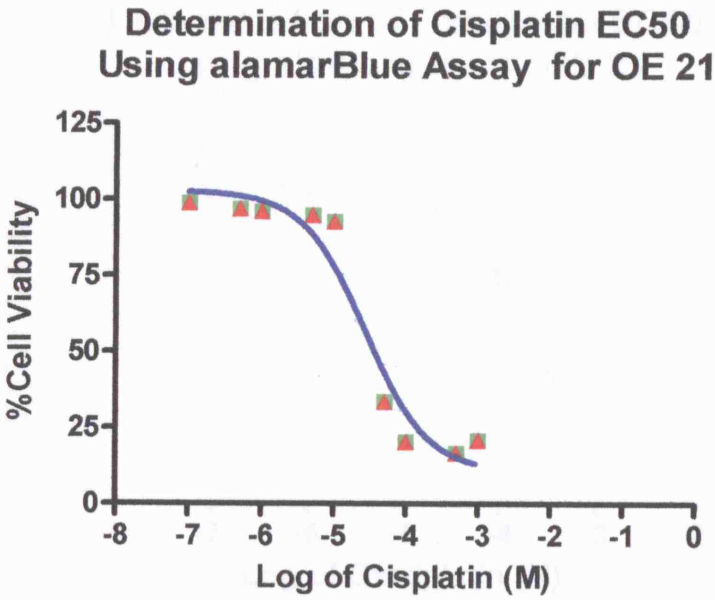


**Figure 8** Concentration effect curve used to determine the EC50 value for cisplatin in OE 21 cells. Plotted on Graph pad Prism Version 4.0, each data set represents the mean of 6 replicates conducted on separate occasions. Prism here takes 100% as maximum and 10% as minimum; the mid point therefore is 45%, which in other words represent 50% of cell viability mark. At this point, the negative log concentration of cisplatin is -4.566M as shown in the graph

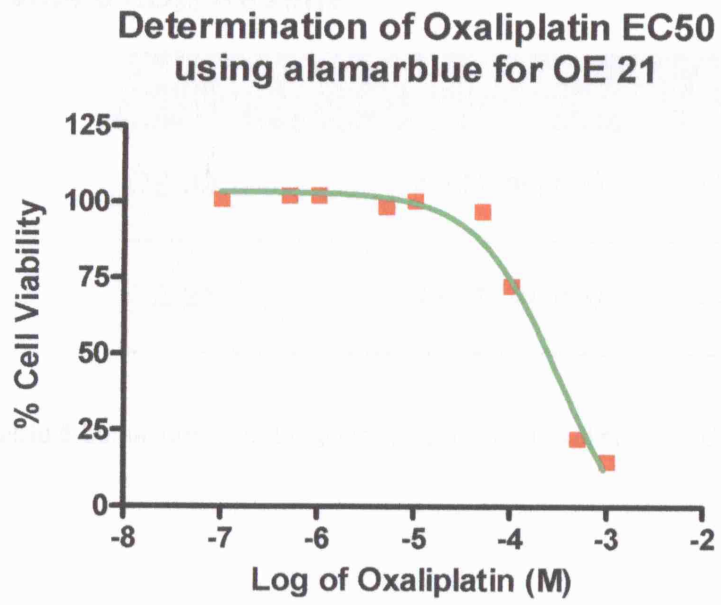


**Figure 9** Concentration effect curve used to determine the EC50 value for oxaliplatin in OE 21 cells. Plotted on Graph pad Prism Version 4.0, each data set represents the mean of 6 replicates conducted on separate occasions. Prism here takes approx 100% as maximum and 12% as minimum; the mid point therefore is 44%, which in other words represent 50% of cell viability mark. At this point, the negative log concentration of cisplatin is -3.488M as shown in the graph

## Table 6 $LD_{50}$ Results

| Cell Type | LD 50 for Cisplatin | LD 50 for Oxaliplatin |
|---|---|---|
| OE 33 | 30μM (approx) | 75μM (approx) |
| OE 21 | 25μM (approx) | 35μM (approx) |

**Table 6** Lethal dose 50 ($LD_{50}$) for cisplatin and oxaliplatin in OE33 and OE21 cell lines

# Chapter 3
## Time Dependent Index

## 3.1 Aims

In the Time Dependent Index (TDI) experiment performed by Arnould et al [160], cytotoxic effects of both cisplatin and oxaliplatin were determined using 1- and 24-h exposures in a panel of six colon cell lines which were found more sensitive to oxaliplatin than to cisplatin. In this experiment the TDI was determined as the ratio between the $IC_{50}$ obtained for the 1-h exposure and the $IC_{50}$ for the 24-h exposure [160].

However in the above experiments oesophageal cells were treated with the $LD_{50}$ dose of cisplatin and oxaliplatin at 2, 4, 6 and 24 hours intervals and TDI was calculated as the ratio of 2, 4 and 6 hours to 24 hour, as 24 hour was the maximum time of exposure and it was assumed that maximum number of cells would be killed by that period of time. Time dependent index experiments were performed to measure the cytotoxicity of cisplatin and oxaliplatin over different time intervals, so that it can be assessed, which drug is more cytotoxic at specific time intervals over 24 hours period in OE 33 & OE 21 cell lines.

## 3.2 Materials & Methods

OE 33 and OE 21 cells were treated with their respective LD50s of cisplatin and oxaliplatin separately. At 2, 4, 6 and 24 hours intervals AlamarBlue™ was added to these cells. Thermo Lab system was used for fluorometric plate readings monitored at 530-560nm excitation wavelength and 590nm emission wavelength using Ascent Software version 2.6 for assessing AlamarBlue™ excitation and emission. TDI evaluations were carried out using Microsoft Excel®

## 3.3 Results for OE 33

Time dependent index was determined with the three independent experiments (figure 10). Time dependent index was also determined as a ratio between the 2, 4 and 6 to 24 hours cisplatin and oxaliplatin as 24 hour was the maximum time of exposure it was assumed that maximum number of cells would be killed in during this exposure time. Thus it was used as standard for the other time periods to assess the number of cell deaths (figure 11). Height of AlamarBlue™ absorbance columns indicated the cell death or in other words the effectiveness of that particular drug in killing the cells.

## Time dependent index in OE33 cells after cisplatin & oxaliplatin Treatment

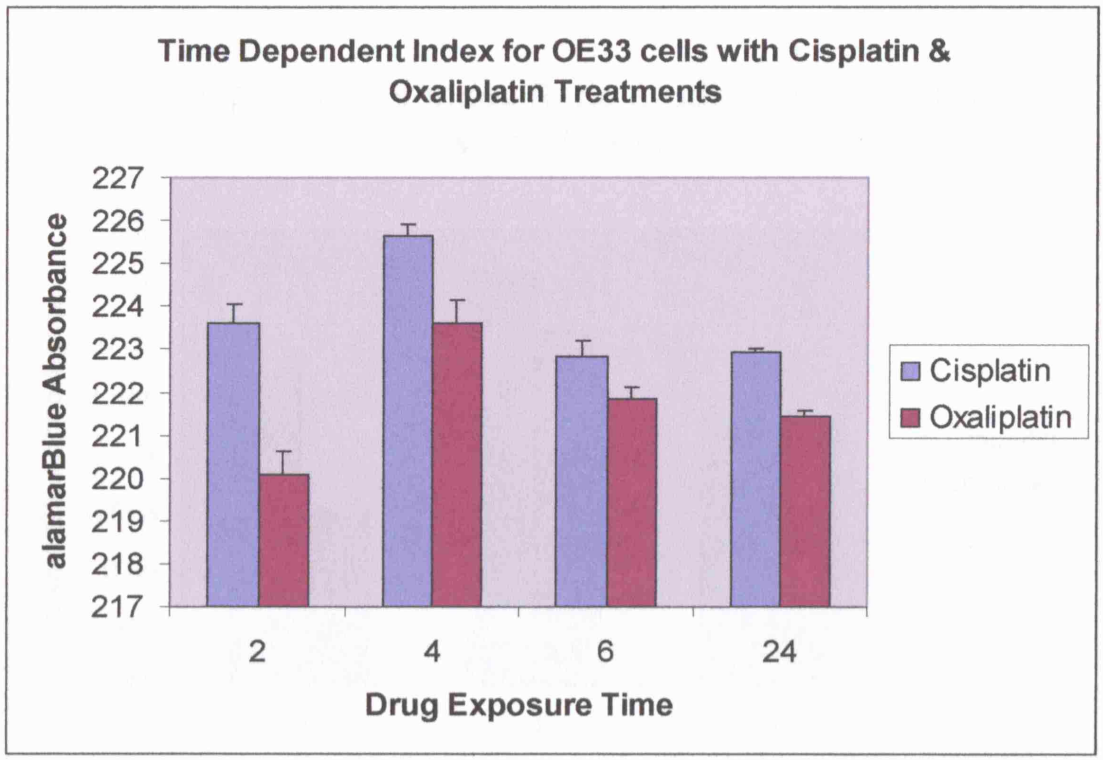


**Figure 10** Time dependent index (TDI) of cisplatin and oxaliplatin in OE 33, cell viability was ascertained using AlamarBlue™ assay. Height of AlamarBlue™ absorbance indicates that at all time intervals cisplatin remained more effective as it killed more cells than oxaliplatin. Results are the mean of three independent experiments

## Time dependent index ratio studies in OE33 for cisplatin & oxaliplatin

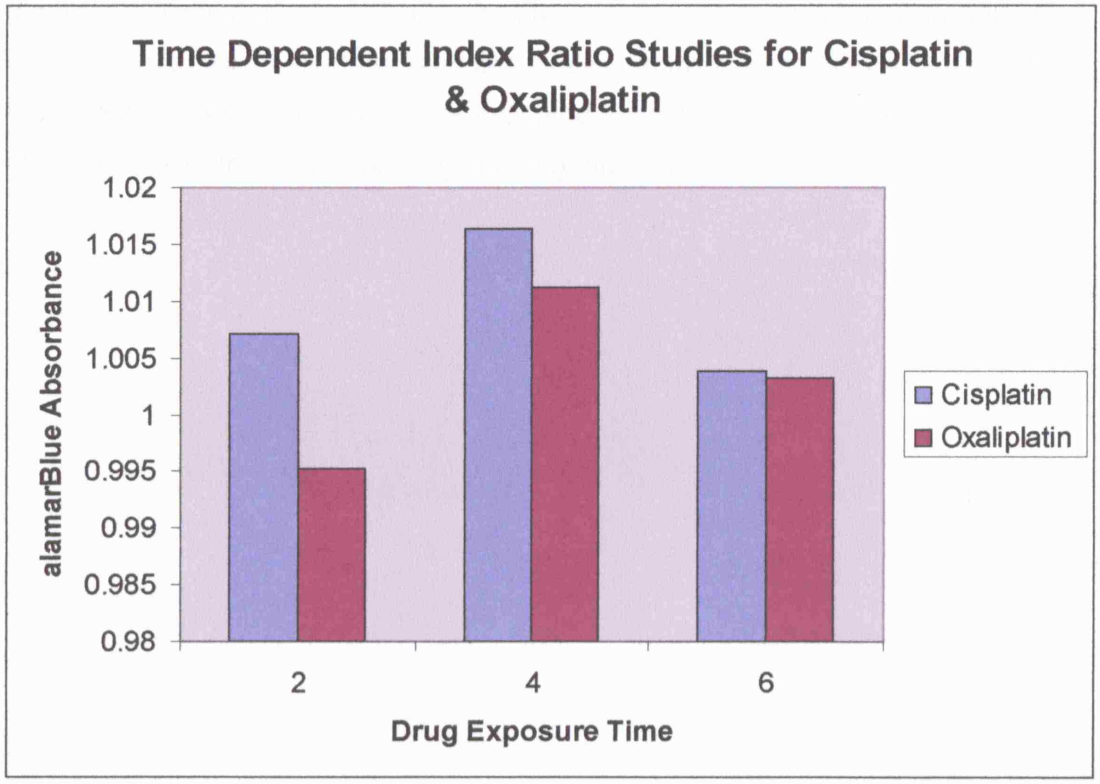


**Figure 11** The ratio between 2, 4 and 6 to 24 hours of cisplatin and oxaliplatin treatments as 24 hours interval which was the maximum time of exposure. Height of the AlamarBlue™ absorbance indicates that at 2 and 4 hours of time intervals cisplatin was more toxic than oxaliplatin but at the 6 hours both were of nearly the same cytotoxicity.

### 3.4 Results for OE 21

Time dependent index was determined with the three independent experiments (figure 12). Time dependent index was also determined as a ratio between the 2, 4, and 6 hours to 24 hours of cisplatin and oxaliplatin treatments (figure 13). Height of AlamarBlue™ absorbance columns indicated the cell death or in other words the effectiveness of the particular drug in killing the cells.

## Time dependent index in OE21 cells after cisplatin & oxaliplatin Treatment

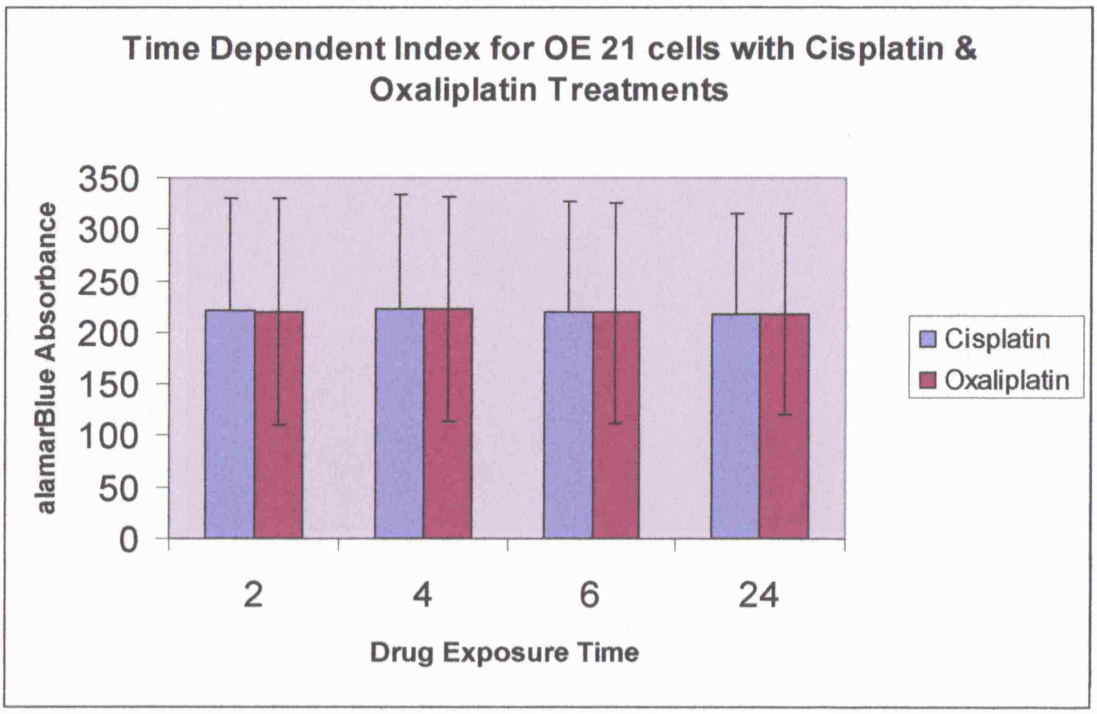


**Figure 12** Time dependent index (TDI) of cisplatin and oxaliplatin in OE 21. Cell viability was ascertained using AlamarBlue™ assay. Results are the mean of three independent experiments

## Time dependent index ratio studies in OE21 for cisplatin & oxaliplatin

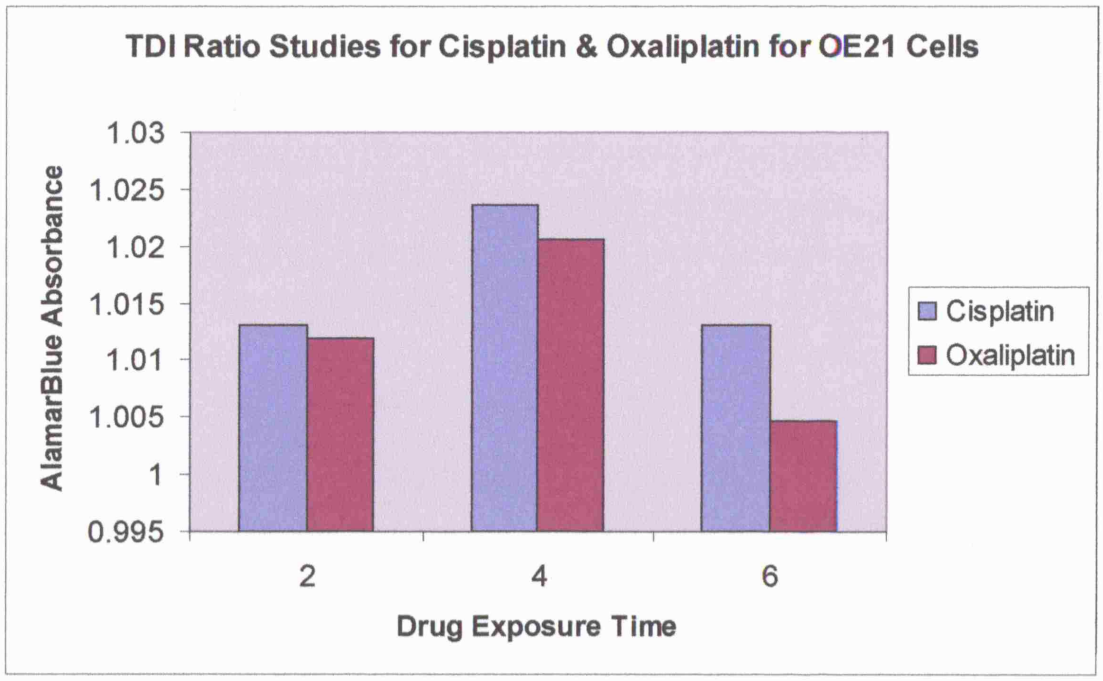


**Figure 13** The ratio between 2, 4 and 6 to 24 hours of cisplatin and oxaliplatin in OE 21 cells. Height of the AlamarBlue™ absorbance indicates that at 2 and 4 hours of time intervals cisplatin and oxaliplatin were having same toxicity level but at 6 hours interval cisplatin seems slightly more cytotoxic than oxaliplatin.

# Chapter 4
## RNA Extraction, RT-PCR and GAPDH

## 4.1 Introduction

To compare the cytotoxicity of cisplatin and oxaliplatin at DNA, mitochondria and apoptotic pathways levels and to evaluate the predictive value of ERCC1 as a potential marker, we assessed the expressions of different genes like ERCC1, XPA, Caspase 3, Bax and Bcl-2. For elaboration of these genes Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR) technique was used. Total RNA extraction was performed from OE33 and OE21 cells after treating them with cisplatin and oxaliplatin at different time intervals.

## 4.2 PCR

Polymerase Chain Reaction (PCR) can amplify a desired DNA sequence of any origin (virus, bacteria, plant, or human) hundreds of millions of times in a matter of hours, a task that would have required several days with recombinant technology. PCR is especially valuable because the reaction is highly specific, easily automated, and capable of amplifying minute amounts of sample. For these reasons, PCR has also had a major impact on clinical medicine, genetic disease diagnostics, forensic science, and evolutionary biology [278].

Reverse transcription (RT) is a mechanism whereby genetic information contained in mRNA is converted back into a double stranded DNA form. The enzyme responsible for this is RNA dependent DNA polymerase called reverse transcriptase. [278]. Process of PCR is based on a specialized polymerase enzyme, which can synthesize a complementary strand to a given DNA strand in a mixture containing the 4 DNA bases and 2 DNA fragments (primers, each about 20 bases long) flanking the target sequence. Repeated heating and cooling cycles multiply the target DNA exponentially, since each new double strand separates to become two templates for further synthesis. In about 1 hour, 20 PCR cycles can amplify the target by a million fold [278]. PCR is closely patterned after the natural principle of DNA replication.

## 4.3 Clinical Value of PCR

The reliability and reproducibility of DNA and RNA techniques are high, because each genetic approach involves qualitative or quantitative assessment of the target towards an internal control (e.g., gene-referee). PCR-related protocols are outstandingly sensitive, and it takes only a few days to develop the assay for each new molecule. Indeed, contrary to antibodies or biochemical kits, all authentic

components of PCR detection (primers, probes) are non-expensive and can be obtained within a short period of time. Finally, nucleic acid tests can be easily applied in a large scale to archival tissue collections.

## 4.4 GAPDH

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a glycolytic enzyme that plays a pivotal role in energy metabolism. It catalyzes the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate in the glycolytic pathway. GAPDH has been referred as a 'house-keeping' protein as its expression remains constant under changing cellular conditions and it acts as an internal control for the PCR reaction [279]

The relative abundance of a transcript in different samples can be estimated by semiquantitative or relative RT-PCR. Typically, the signal from the RT-PCR product is normalized to the signal from an internal control included in all samples and amplified at the same time as the target. The normalized data from different samples can then be compared. Transcripts of housekeeping genes such as GAPDH or β-actin are frequently chosen as internal controls because they are abundantly expressed at relatively constant rates in most cells [279].

## 4.5 Materials and Methods

More than $1x10^6$ cells/well were used for both cell lines to have a better yield of RNA and treated with the respective LD 50 of cisplatin and oxaliplatin at 2, 4, 6 and 24 hours. At the end of each time interval cells were washed away with cold PBS three times and then small amount of trypsin was used to separate the cells from the plate surface. The cell pallets got after centrifuging the cells, were used for RNA extraction. RNeasy® Mini Kit form QIAGEN™ was used for RNA Extraction. Protocol for animal cells was followed from the QIAGEN™ RNA extraction handbook. GAPDH RT-PCR for OE 33 and OE 21 was performed using One-Step RT-PCR Kit ® from QIAGEN ® and specific primers for GAPDH (forward 5'-AGT ATG ATG ACA TCA AGA AGG and reverse 5'-ATG GTA TTC AAG AGA GTA GGG). Protocol for QIAGEN One-Step RT-PCR Kit was used. For one PCR reaction following components and their quantities were used.

5 x QIAGEN OneStep RT-PCR buffer (12.5mM $MgCl_2$) = 10µl,
5 x Q-solution =10µl,
dNTP Mix (containing 10mM of each dNTP) = 2µl,
QIAGEN OneStep RT-PCR Enzymes Mix =2µl,

Primers Reverse 0.5µl and Forward 0.5µl
Conditions for PCR cycle were denaturation 1 min at 94°C, hybridisation 1 min at 60°C and extension 1 min 30 s at 72 °C for 30 cycles.
The Band heights for GAPDH at different time intervals was calculated using Syngene® Gene Tools® programme and Microsoft Excel® programmes

## 4.6 RNA Extraction Results for OE 33 & 21

The integrity of the RNA for OE33 (figure 14) and OE21 (figure 15) were checked by performing gel electrophoresis. It is clear that yields are significantly higher. Ratios are around the ideal value of 2.0, suggesting qualitatively pure RNA yield.

## RNA Extraction from OE 33 cell after Cisplatin & Oxaliplatin Treatment

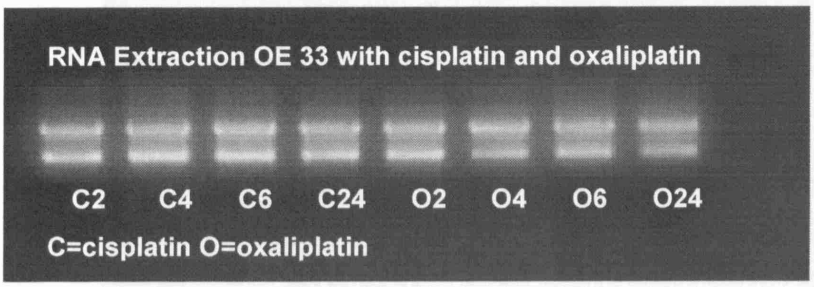


**Figure 14** Expression of RNA (2% agarose gel in TBE Buffer) in OE33 cells following treatment with cisplatin and oxaliplatin for 2, 4, 6 or 24 hours. The 18S and 28S ribosomal RNA bands are clearly visible in the intact RNA sample. The reactive ribosomal bands appeared as sharp bands on the gel. The intensity of the 28S ribosomal RNA band appeared twice of the 18S band of ribosomal RNA.

## RNA Extraction from OE 21 cell after Cisplatin & Oxaliplatin Treatment

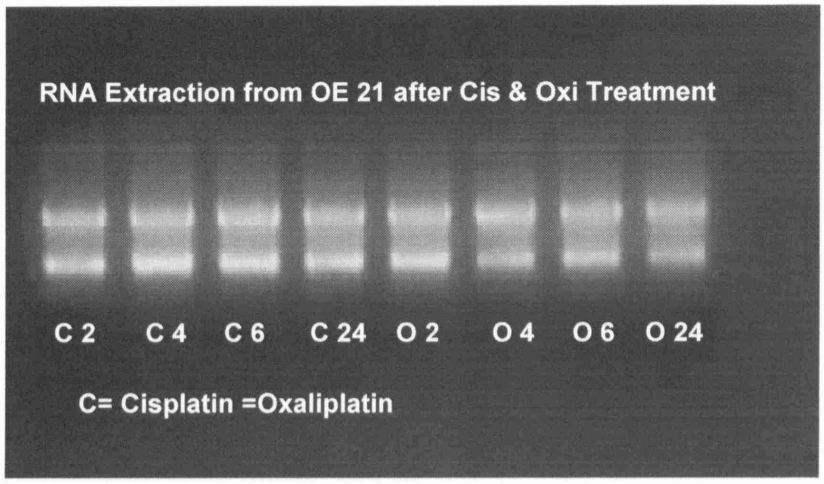


**Figure 15** Expression of RNA (2% agarose gel in TBE Buffer) in OE 21 cells following treatment with cisplatin and oxaliplatin for 2, 4, 6 or 24 hours. The 18S and 28S ribosomal RNA bands are clearly visible in the intact RNA sample. The reactive ribosomal bands appeared as sharp bands on the gel. The intensity of the 28S ribosomal RNA band appeared twice of the 18S band of ribosomal RNA.

## 4.7 OE33 and OE21 GAPDH Results

Size of amplicons for GAPDH was 220 base pair. It was expressed equally in all treatment groups for OE 33 (figure 16) & OE 21 (figure 17) cells. Calculation of band height for OE 33 & 21 cell lines demonstrated a fairly equal expression of RNA in all treatment schemes (figure 18) for OE 33 and (figure 18) for OE21

## GAPDH Expression in OE33 Cells after Cisplatin & Oxaliplatin Treatment

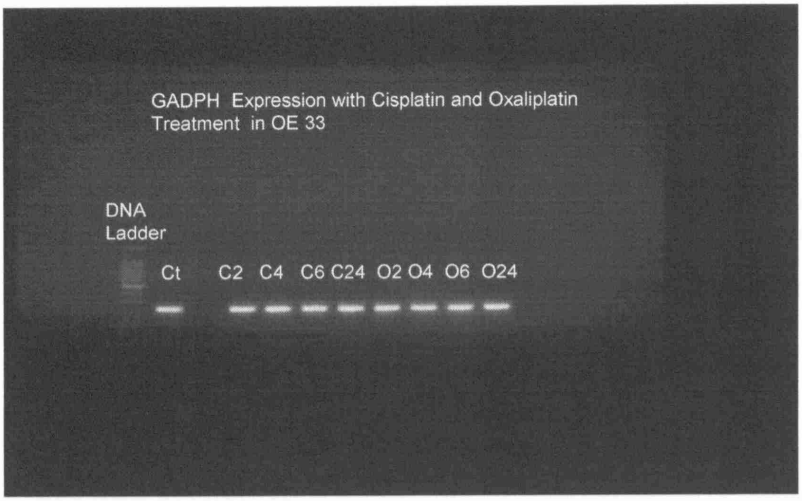


**Figure 16** GAPDH Gene expression (2% agarose gel) in OE33 cells following cisplatin and oxaliplatin treatment at 2, 4, 6 or 24 hours.
C = Cisplatin O = oxaliplatin.

## GAPDH Expression in OE 21 Cells after Cisplatin & Oxaliplatin Treatment

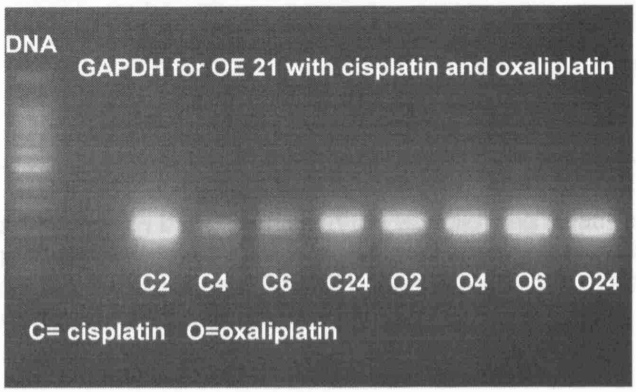


**Figure 17** GAPDH Gene expression of GAPDH (2% agarose gel) in OE 21 cells following cisplatin and oxaliplatin treatment at 2, 4, 6 or 24 hours.
C = Cisplatin O = oxaliplatin.

## GAPDH Expression in OE 33 Cells after Cisplatin & Oxaliplatin Treatment

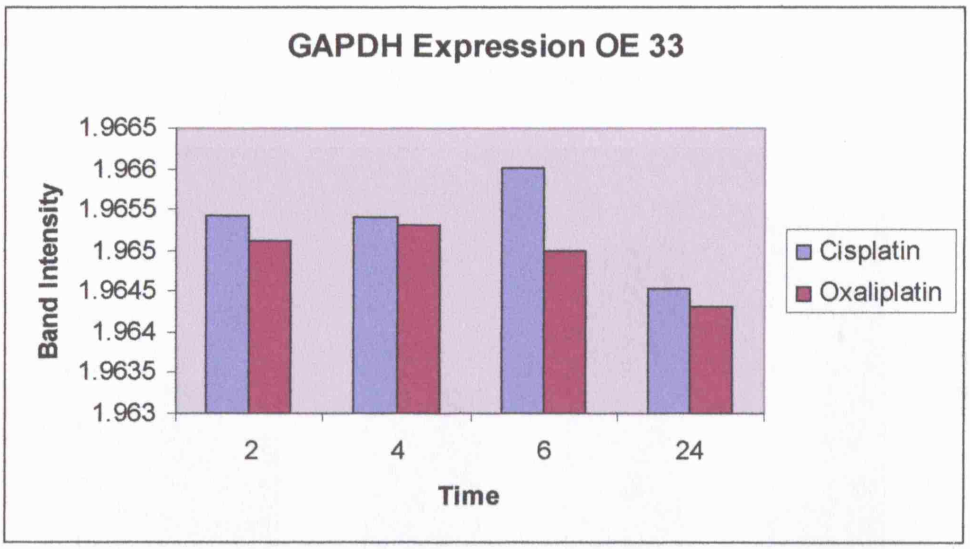


**Figure 18** GAPDH band heights in OE 33 following treatment with cisplatin and oxaliplatin calculated by Syngene® Gene Tools® and Microsoft Excel® programmes, which demonstrate the significant GAPDH gene expressions at all treatment intervals with cisplatin and oxaliplatin.

## GAPDH Expression in OE 21 Cells after Cisplatin & Oxaliplatin Treatment

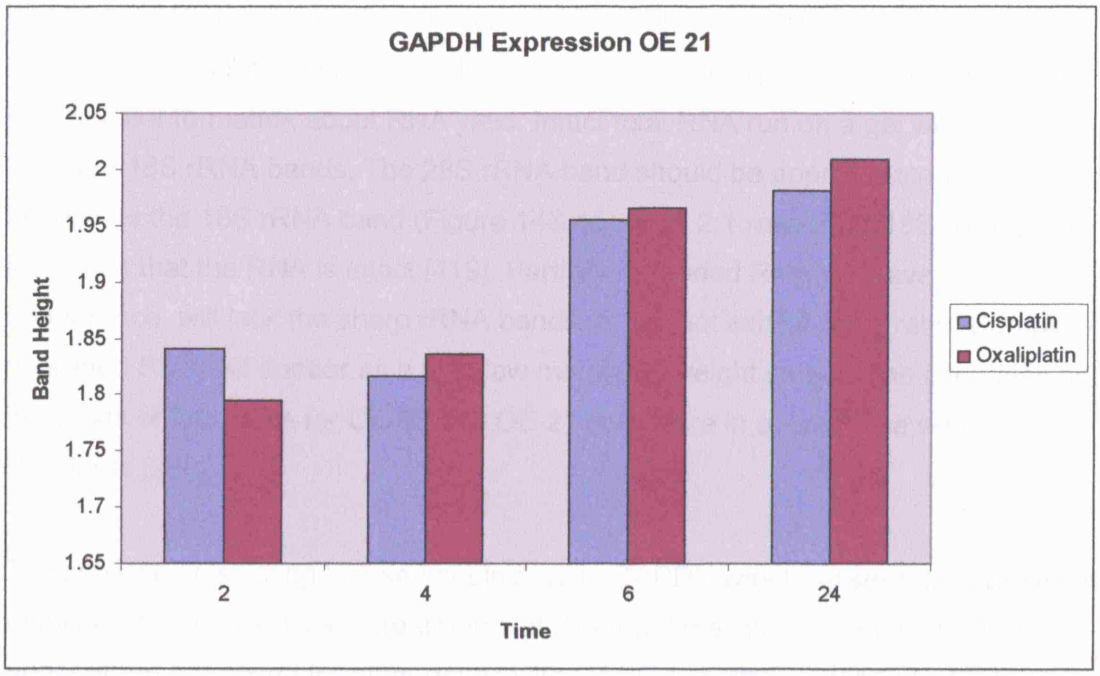


**Figure 19** GAPDH band heights in OE 21 following treatment with cisplatin and oxaliplatin calculated by Syngene® Gene Tools® and Microsoft Excel® programmes, which demonstrate the significant GAPDH gene expressions at all treatment intervals with cisplatin and oxaliplatin.

## 4.8 Discussion

The extraction of total RNA is an important step in RT-PCR, since the quantity and quality of RNA is essential for a successful RT-PCR. The overall quality of an RNA preparation may be assessed by electrophoresis on an agarose gel. This will also give some information about RNA yield. Intact total RNA run on a gel will have sharp 28S and 18S rRNA bands. The 28S rRNA band should be approximately twice as intense as the 18S rRNA band (Figure 14& 15). This 2:1 ratio (28S:18S) is a good indication that the RNA is intact [419]. Partially degraded RNA will have a smeared appearance, will lack the sharp rRNA bands, or will not exhibit a 2:1 ratio. Completely degraded RNA will appear as a very low molecular weight smear. The integrities and the yields of total RNA for OE 33 and OE 21 cells were in accordance with the standards [280]

The purpose of isolating 'house keeping' gene GAPDH was to assess its expression with cisplatin and oxaliplatin treatments at different time intervals, so that RT-PCR work can be correlated for other genes with GAPDH levels. Expression of GAPDH also demonstrates that RNA in any sample is intact and can safely be used for any RT-PCR work [279]. GAPDH is referred as a 'house-keeping' protein since its expression remains relatively abundant and constant under changing cellular conditions and it acts as an internal control for the PCR reaction in most cells. Results of GAPDH work demonstrated that it was significantly expressed on agorose gel demonstrated by Syngene® Gene Tools® in both cell line OE 33 & 21 with cisplatin and oxaliplatin treatments at different time intervals. Band heights of GAPDH were analysed by using Syngene® Gene Tools® and Microsoft Excel® programmes (figs 16 & 17), which also confirmed that GAPDH was significantly expressed with cisplatin and oxaliplatin at all treatment intervals in both cell lines (figs 18 & 19)

# Chapter 5
## Experiments for ERCC1 and XPA

## 5.1 Aims

It has been demonstrated that a high level of ERCC1 expression could induced a greater removal of cisplatin DNA adducts and consequently, lowers its cytotoxic effect. The level of ERCC1 expression has also been reported to predict cisplatin sensitivity in gastric, ovarian and Non Small Cell Lung cancers (NSCLC) [98].

In addition to ERCC1, cisplatin sensitivity is also highly dependent on the DNA repair protein XPA. XPA is a critical player in DNA damage recognition and has identified as critical to cell's ability to respond to cisplatin insult. Extremely low levels of XPA and to a lesser degree ERCC1 were found responsible for the pronounced cisplatin sensitivity in Non Seminoma cell line. Elevated levels of XPA were found in cisplatin-resistant tumours [161].
The aims of these experiments were to assess the ERCC1 expression levels after cisplatin and oxaliplatin treatments so that predictive value of ERCC1 in OE 33 and 21 cells can be assessed as it displayed previously in other carcinomas. Similarly experiments were performed to see the effects on XPA expression after cisplatin and oxaliplatin treatments.

## 5.2 Materials & Methods

### RT-PCR for ERCC1

To assess the ERCC1 gene expression, RT-PCR was performed on the previously extracted RNA by using specific primers and One-Step RT-PCR Kit ® from QIAGEN®. For one PCR reaction following components and their quantities were used. 5 x QIAGEN OneStep RT-PCR buffer (12.5mM $MgCl_2$) = 10µl,
5 x Q-solution =10µl, dNTP Mix (containing 10mM of each dNTP) = 2µl,
QIAGEN OneStep RT-PCR Enzymes Mix =2µl, Primers Reverse 0.5µl and Forward 0.5µl.
PCR conditions were denaturation 1 min at 94°C, hybridisation 1 min at 60°C and extension 1 min 30 s at 72 °C for 30 cycles.
The Band heights for ERCC1 at different time intervals were calculated using Syngene® Gene Tools® and Microsoft Excel® programmes. Ratio between ERCC1 and GAPDH values were obtained using Syngene Gene Tools® programme.

### RT-PCR for XPA

To assess the XPA gene expression, RT-PCR was performed on the previously extracted RNA by using specific primers and One-Step RT-PCR Kit ® from

QIAGEN®. For one PCR reaction following components and their quantities were used.

5 x QIAGEN OneStep RT-PCR buffer (12.5mM $MgCl_2$) = 10µl,

5 x Q-solution =10µl,

dNTP Mix (containing 10mM of each dNTP) = 2µl,

QIAGEN OneStep RT-PCR Enzymes Mix =2µl,

Primers Reverse 0.5µl and Forward 0.5µl

PCR conditions were denaturation 1 min at 94°C, hybridisation 1 min at 60°C and extension 1 min 30 s at 72 °C for 30 cycles.

The Band intensities for XPA at different time intervals were calculated using Syngene® Gene Tools® and Microsoft Excel® programmes. Correlation between ERCC1 and XPA values was obtained by using Syngene Gene Tools® and Microsoft Excel® programmes.

## 5.3 Results of ERCC1 expression in OE 33

Low levels of ERCC1 were seen at 2 and 4 hors and fairly significant levels were seen at 6 hours while at 24 hours very low levels were detected with cisplatin treatment. However, its levels following oxaliplatin treatment were higher at 2 hours, increased at 4 hours and reduced slightly at 6 and 24 hours (figure 38). These values were confirmed by measuring the band heights as well (figure 39).

ERCC1/GAPDH ratio demonstrated that ERCC1 is explicitly expressed in cells treated with oxaliplatin. In cisplatin treated cells, only small amounts of ERCC1 were seen in the initial hours (figure 40).

## ERCC1 Expression in OE 33 cells after cisplatin & oxaliplatin Treatment


**Figure 20** ERCC1Gene expression (2% agarose gel) in OE33 cells following treatment with cisplatin and oxaliplatin at 2, 4, 6 and 24 hours intervals.
C = Cisplatin O = oxaliplatin.

## ERCC1 band heights after cisplatin & oxaliplatin Treatment in OE 33 cells

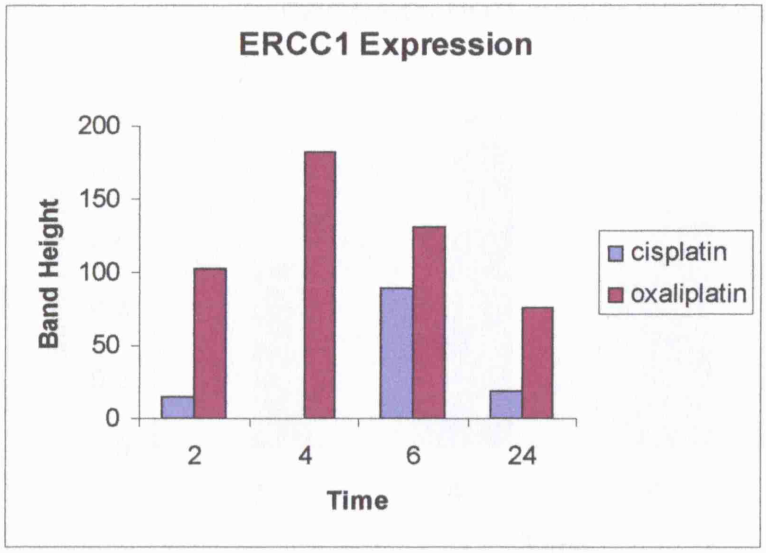


**Fig 21** The Band heights of ERCC1 at different time intervals calculated using Syngene® Gene Tools® and Microsoft Excel® programmes which demonstrate nearly undetectable levels of ERCC1 gene expression with cisplatin treatment at 2, 4 and 24 hours however, at 6 hours it is significantly expressed. ERCC1gene expressions are explicitly high at all treatment intervals with oxaliplatin.

## Ratio of ERCC1/GAPDH for OE 33 cells

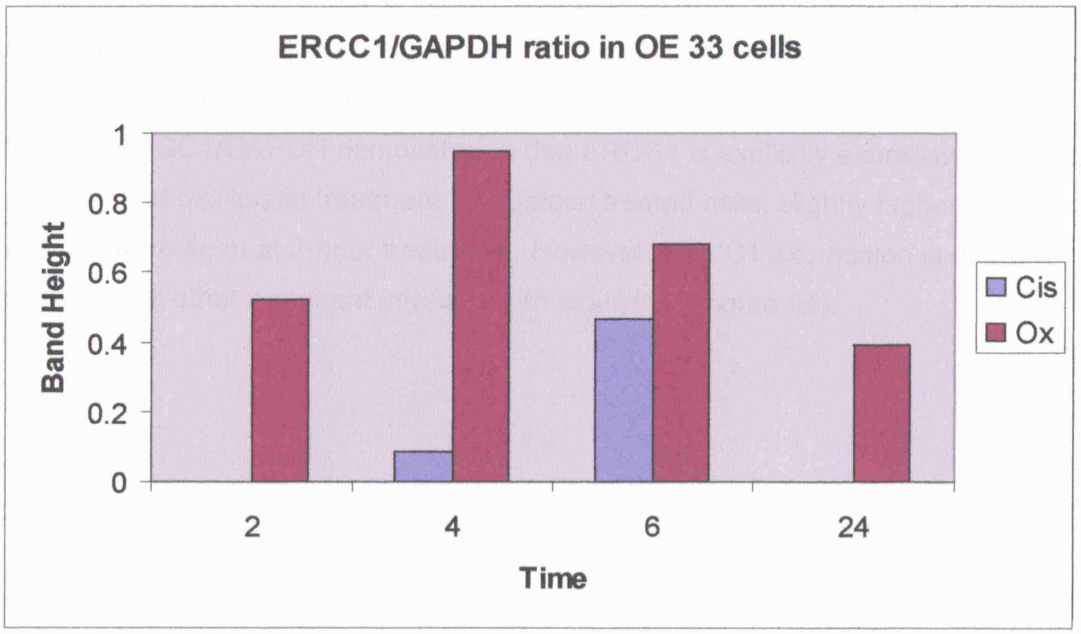


**Fig 22** Ratio of ERCC1/GAPDH with cisplatin and oxaliplatin treatment at 2, 4, 6 and 24 hours intervals calculated using Syngene® Gene Tools® and Microsoft Excel® programmes, which demonstrate nearly undetectable levels of ERCC1 gene expression with cisplatin treatment at 4 hours and significantly expressed at 6 hours. ERCC1gene expressions are considerably high at all treatment intervals with oxaliplatin.

### 5.4 Results of ERCC1 expression in OE 21

ERCC1 was expressed mostly equally with both cisplatin and oxaliplatin (figure 23). ERCC1 band height analysis demonstrated that it is approximately equally expressed with both cisplatin and oxaliplatin treatments (figures 24 & 25).
Ratio of ERCC1/GAPDH demonstrated that ERCC1 is explicitly expressed with both cisplatin and oxaliplatin treatment. In cisplatin treated cells, slightly higher amounts of ERCC1 were seen at 2-hour treatment. However, ERCC1 expression is at same height for all other treatment intervals with oxaliplatin (figure 25).

## ERCC1 Expression in OE 21 cells after cisplatin & oxaliplatin Treatment

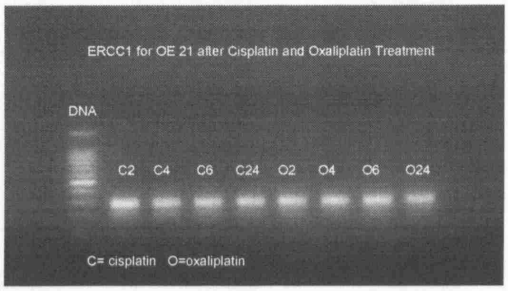


**Fig 23** ERCC1Gene expression (2% agarose gel) in OE21 cells following treatment with cisplatin and oxaliplatin at 2, 4, 6 and 24 hours intervals
C = Cisplatin O = oxaliplatin.

## ERCC1 band heights after cisplatin & oxaliplatin Treatment in OE 21 cells

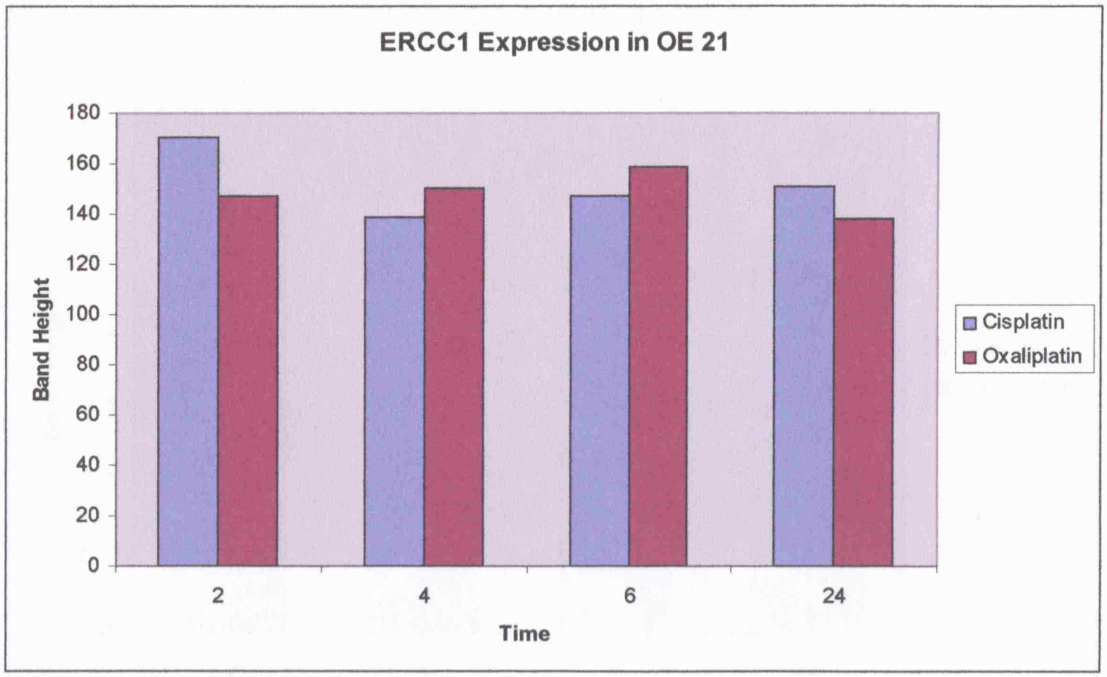


**Fig 24** The Band heights of ERCC1 at different time intervals calculated using Syngene® Gene Tools® and Microsoft Excel® programmes which demonstrate significantly high levels of ERCC1 with both cisplatin and oxaliplatin treatments at all time intervals.

## Ratio of ERCC1/GAPDH for OE 21 cells

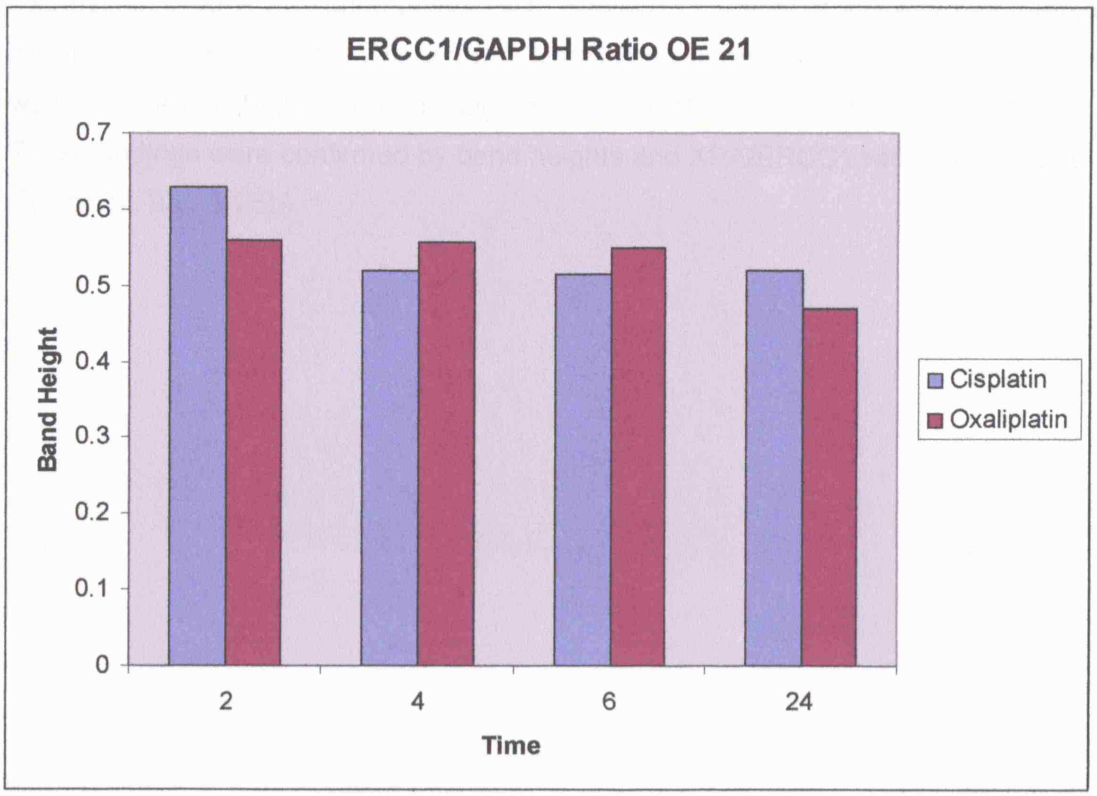


**Fig 25** ERCC1/GAPDH ratios with cisplatin and oxaliplatin treatment at 2, 4, 6 and 24 hours intervals calculated using Syngene® Gene Tools® and Microsoft Excel® programmes which demonstrate significantly high levels of ERCC1 with both cisplatin and oxaliplatin treatments at all time intervals

## 5.5 Results of XPA expression

Expression of XPA was undetectable with cisplatin treatment at all treatment intervals except 24 hours treatment where it was significantly expressed; while its expression was considerably high with oxaliplatin treatment at all time intervals (figure 26). These findings were confirmed by band heights and XPA/ERCC1 ratio calculations (figure 27, 28a & 28b)

## XPA Expression in OE 33 cells after cisplatin & oxaliplatin Treatment

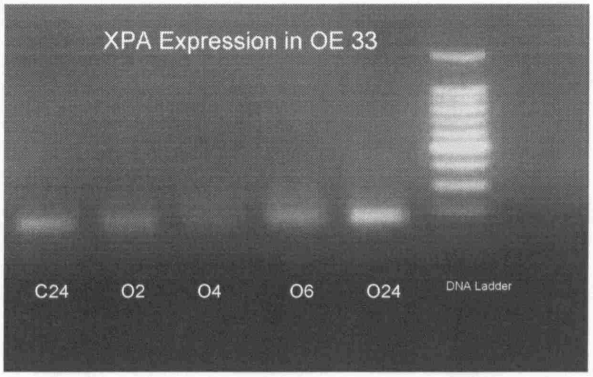


**Figure 26** XPA Gene expression (2% agarose gel) in OE33 cells following treatment with cisplatin and oxaliplatin at 2, 4, 6 or 24 hours intervals
C= cisplatin and O= oxaliplatin

## Band heights of XPA after cisplatin & oxaliplatin Treatment in OE 33

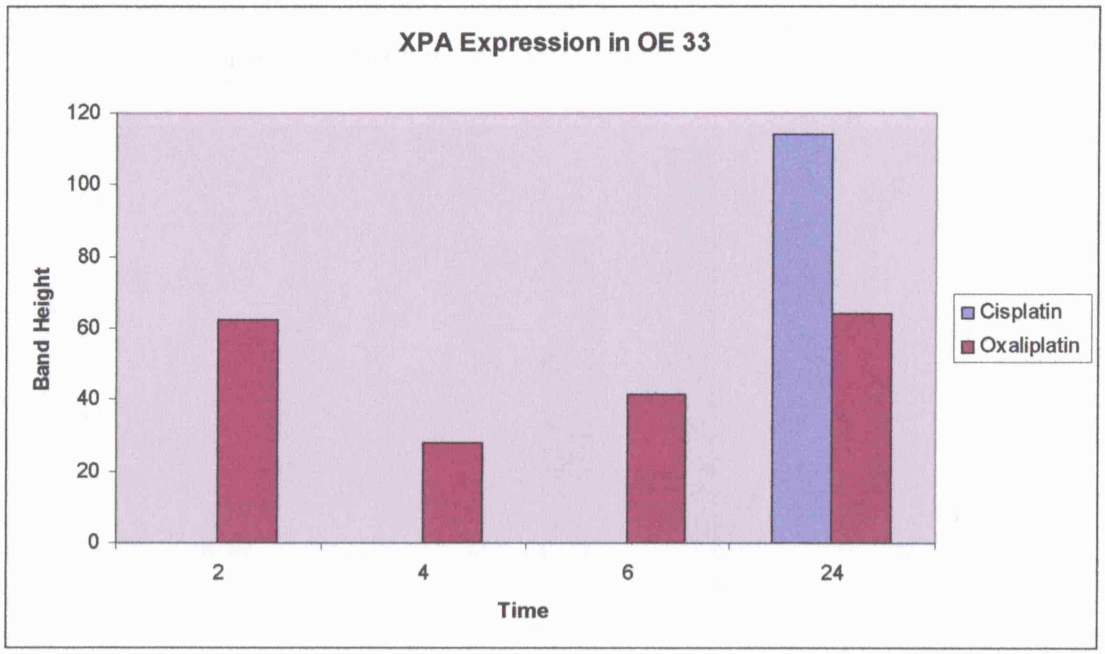


**Fig 27** The Band heights for XPA at different time intervals calculated using Syngene® Gene Tools® and Microsoft Excel® programmes which demonstrate that XPA levels are undetectable after cisplatin treatments at 2, 4 and 6 hours while at 24 hours it is significantly expressed. However, XPA is expressed considerably at all time intervals after oxaliplatin treatment.

## Band heights of XPA & ERCC1 after cisplatin treatment in OE 33 cells

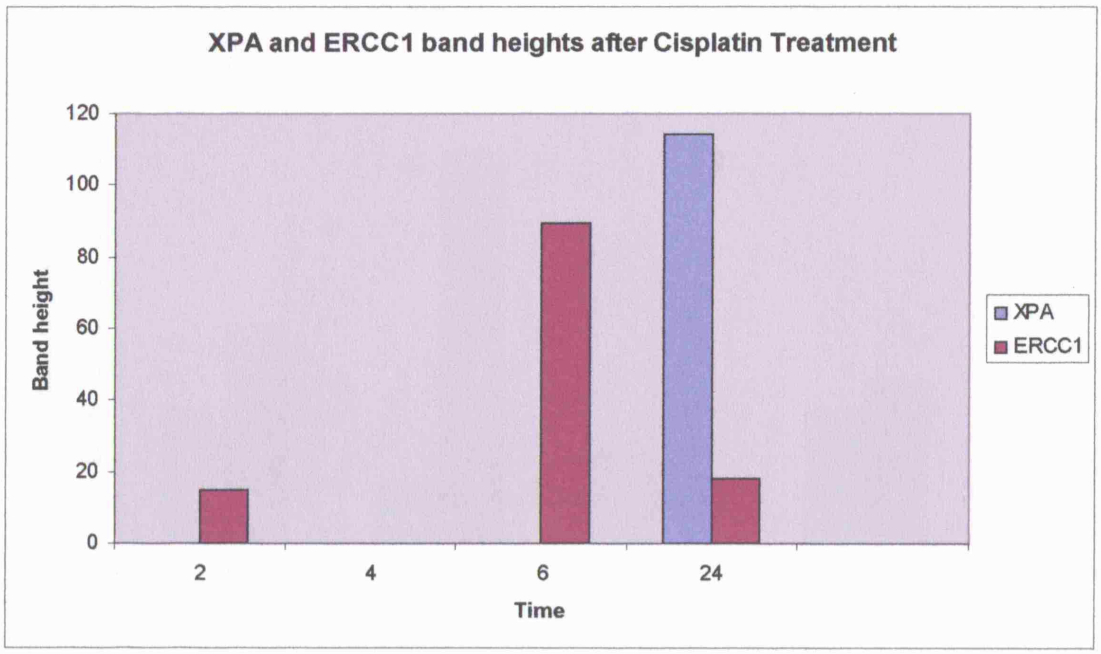


**Fig 28a** The Band heights for XPA at different time intervals calculated using Syngene® Gene Tools® and Microsoft Excel® programmes which demonstrate that no significant XPA and ERCC1 expression was detectable after cisplatin treatment at 2 and 4 hours intervals. Significant levels of ERCC1 were present at 6 hours and XPA at 24 hours intervals.

## Band heights of XPA and ERCC1 after oxaliplatin Treatment in OE 33 cells

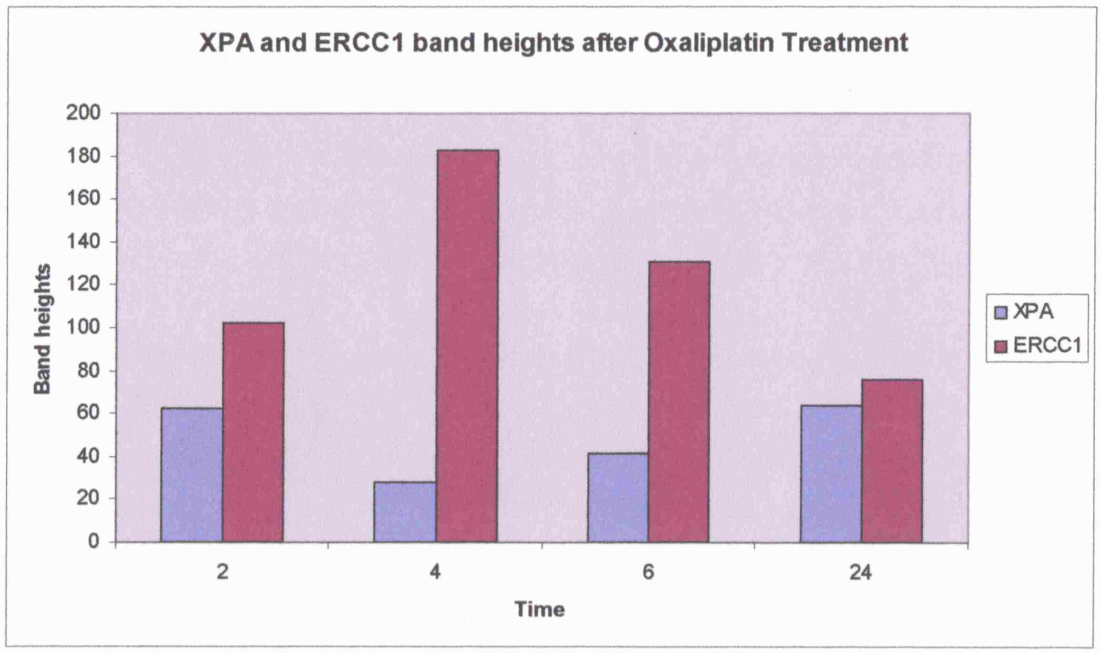


**Fig 28b** The Band heights for XPA at different time intervals calculated using Syngene® Gene Tools® and Microsoft Excel® programmes which demonstrate that significant levels of XPA and ERCC1 expression were detectable after oxaliplatin treatment at all treatment intervals.

# Chapter 6
## Experiments for Bcl-2, Bax and Caspase 3 Expression

## 6.1 Aims

The most prominent proteins involved in the regulation of the mitochondrial apoptosis pathway are Bcl-2 family of proteins. Bax and Bcl-2 gene expression indicates that mitochondrion is taking active part in the induction of apoptosis. Cisplatin-induced apoptosis in NER deficient cells appears to be controlled by the mitochondrial apoptotic pathway which appears to be regulated by changes in the Bax/Bcl-2 expression ratio, as a decline of Bcl-2 protein level was observed upon cisplatin treatment in experiments of Dunkern and colleagues [161]

Heterodimerization of Bax and Bcl-2 are supposed to generate pores in the outer mitochondrial membrane, thus releasing cytochrome C [281] which in combination with other cytoplasmic proteins and pro-caspase-9 creates apoptosome complex [282]. This complex generates the active caspase-9 which in turn activates the apoptosis executioner caspase-3. This indicates that caspases were triggered by non-repaired DNA damage, very likely via down-regulation of Bcl-2.

Therefore the aims of these experiments were to see the effects on Bax and Bcl-2 expression in OE33 and OE21 cells after cisplatin and oxaliplatin treatments. Furthermore experiments were also performed to evaluate the effects of cisplatin and oxaliplatin treatments on expression of caspase 3 above cell lines.

## 6.2 Material & Methods

### RT-PCR for Bax and Bcl-2

To assess the Bax and Bcl-2 gene expression, RT-PCR was performed on the previously extracted RNA by using specific primers and One-Step RT-PCR Kit ® from QIAGEN®. For one PCR reaction following components and their quantities were used.

5 x QIAGEN OneStep RT-PCR buffer (12.5mM $MgCl_2$) = 10µl,
5 x Q-solution =10µl,
dNTP Mix (containing 10mM of each dNTP) = 2µl,
QIAGEN OneStep RT-PCR Enzymes Mix =2µl,
Primers Reverse 0.5µl and Forward 0.5µl
PCR conditions were denaturation 1 min at 94°C, hybridisation 1 min at 60°C and extension 1 min 30 s at 72 °C for 30 cycles. The Band intensities for Bax and Bcl-2 at different time intervals were calculated using Syngene® Gene Tools® and Microsoft

Excel® programmes. Ratio between caspase 3 and GAPDH values was obtained using Syngene Gene Tools® programme.

**RT-PCR for Caspase 3**

To assess the caspase 3 gene expression, RT-PCR was performed on the previously extracted RNA by using specific primers and One-Step RT-PCR Kit ® from QIAGEN®. For one PCR reaction following components and their quantities were used.

5 x QIAGEN OneStep RT-PCR buffer (12.5mM $MgCl_2$) = 10µl,

5 x Q-solution =10µl,

dNTP Mix (containing 10mM of each dNTP) = 2µl,

QIAGEN OneStep RT-PCR Enzymes Mix =2µl,

Primers Reverse 0.5µl and Forward 0.5µl

PCR conditions were denaturation 1 min at 94°C, hybridisation 1 min at 60°C and extension 1 min 30 s at 72 °C for 30 cycles. The Band intensities for caspase 3 at different time intervals were calculated using Syngene® Gene Tools® and Microsoft Excel® programmes. Ratio between caspase 3 and GAPDH values was obtained using Syngene Gene Tools® programme. Correlation of caspase 3 and ERCC1 values was performed using Microsoft Excel®

The Bcl-2 family of proteins play a key role in keeping a balance between pro and anti apoptotic stimuli. Bax induces cytochrome c release while Bcl-2 stops it.

Bax and Bcl-2 gene expression indicates that mitochondrion is taking active part in the induction of apoptosis. The aims of these experiments were to see the effects on Bax and Bcl-2 gene expression and assess the pro and anti apoptosis ratio of these genes after cisplatin and oxaliplatin treatments

## 6.3 Results of Bcl-2 and Bax expression in OE 33 cells

Band heights analysis calculated demonstrated that after cisplatin treatment Bax was significantly expressed at all time intervals while Bcl-2 was expressed considerably less at all time intervals, sizes of the amplicons were Bax 95 & Bcl-2 235bp (figures 29, 30 & 31) After oxaliplatin treatment Bax was less significantly expressed at 2, 4 and 24 hours, while at 6 hours its expression was considerably high. However, Bcl-2 was significantly expressed at 2, 4 and 6 hour while less significantly expressed at 24 hours interval (figures 29, 30 & 32)

Ratio of Bax/Bcl-2 demonstrated that Bax was significantly expressed after cisplatin treatment at 2, 4 and 6 hours, while at 24 hours its expression was considerably low.

Expression of Bcl-2 was considerably less than Bax at all treatment intervals after oxaliplatin treatment (figure 33).

Bax & Bcl-2/GAPDH ratio after cisplatin treatment demonstrated that Bax was quite significantly expressed at 2, 4, 6 and 24 hours; while Bcl-2 was less significantly expressed at all treatment intervals (figure 34).

Bax & Bcl-2/GAPDH ratio after oxaliplatin treatment demonstrated that Bcl-2 was significantly expressed at 2, 4, 6 and 24 hours. Expression of Bax was significantly low at 2, 4 and 24 hours but at 6 hours interval it was significantly expressed (fig 35)

## Bcl-2 expression in OE 33 cells after cisplatin & oxaliplatin Treatment

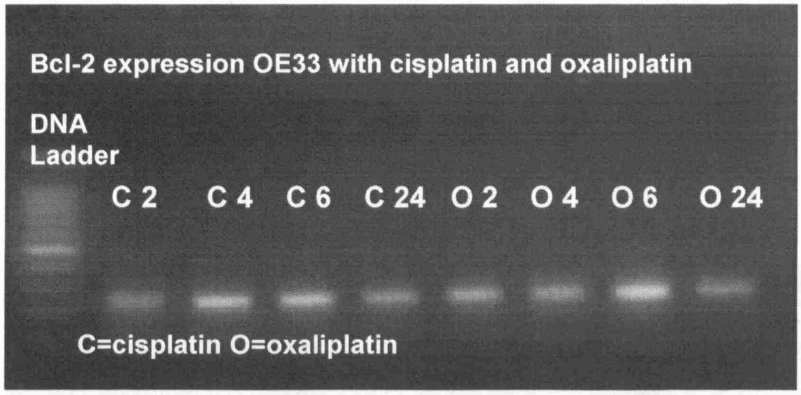


**Figure 29** Gene expression of bcl-2 (2% agarose gel) in OE33 cells following treatment with cisplatin and oxaliplatin at 2, 4, 6 or 24 hours intervals.
C= Cisplatin, O= Oxaliplatin

## Bax expression in OE 33 cells after cisplatin & oxaliplatin Treatment

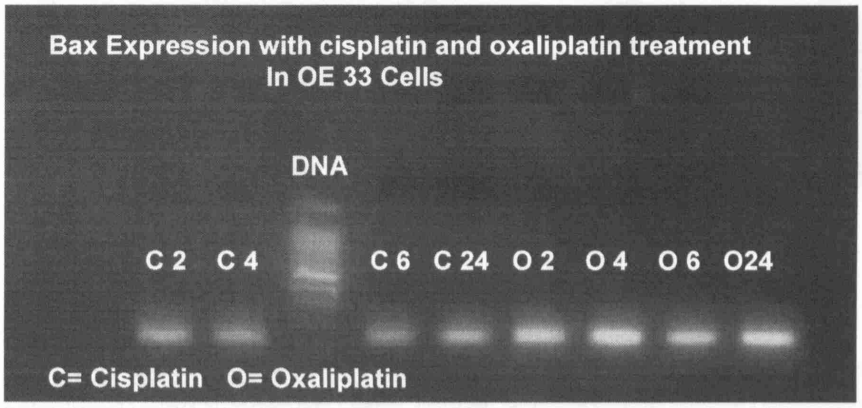


**Figure 30** Gene expression of Bax (2% agarose gel) in OE33 cells following treatment with cisplatin and oxaliplatin at 2, 4, 6 or 24 hours intervals.
C=Cisplatin, O=Oxaliplatin

## Bax and Bcl-2 Band heights in OE 33 cells after cisplatin Treatment

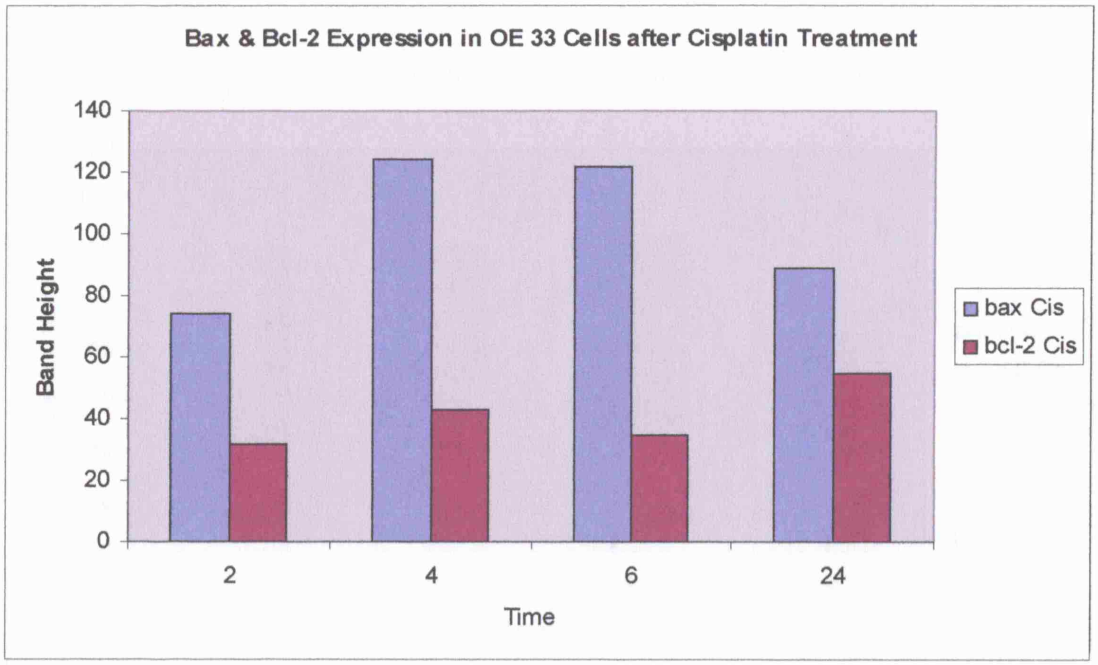


**Figure 31** Band heights of Bax and Bcl-2 with cisplatin treatment at different time intervals calculated using Syngene® Gene Tools® and Microsoft Excel® programmes, which demonstrated that Bax was significantly expressed after cisplatin treatment at all time intervals, while Bcl-2 was expressed considerably less at all time intervals.

## Bax and Bcl-2 Band heights in OE 33 cells after oxaliplatin Treatment

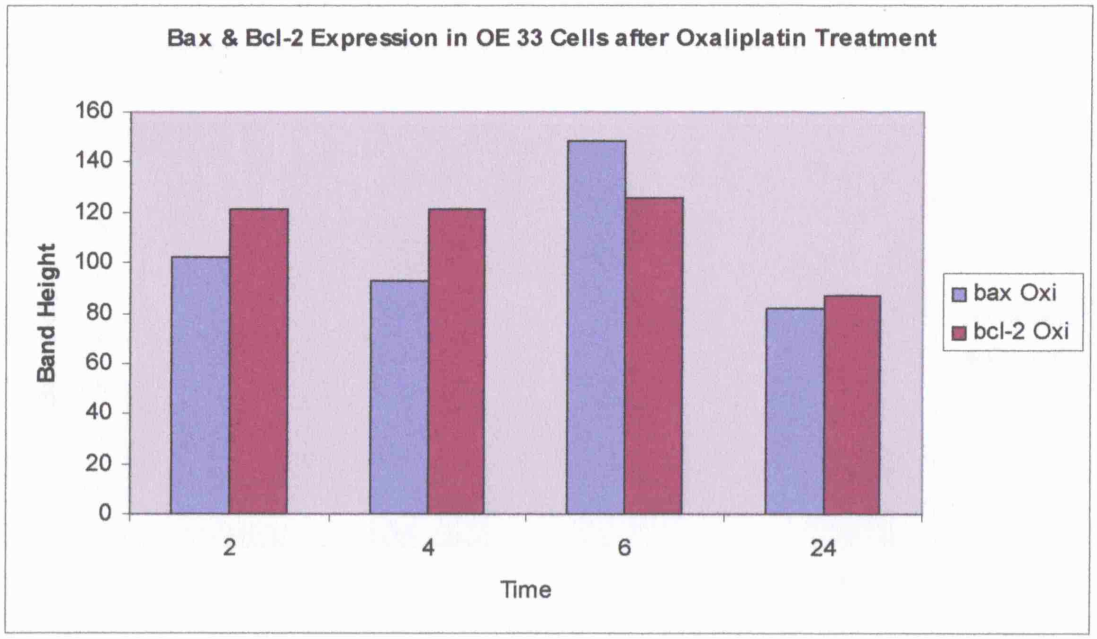


**Figure 32** Band heights of Bax and Bcl-2 with oxaliplatin treatment at different time intervals calculated using Syngene® Gene Tools® and Microsoft Excel® programmes, which demonstrated that Bax was less significantly expressed after oxaliplatin treatment at 2, 4 and 24 hours, while at 6 hours its expression was considerably high. However, Bcl-2 was significantly expressed at 2, 4 and 6 hour but less significantly at 24 hours after oxaliplatin treatment

## Ratio of Bax/Bcl-2 in OE 33 cells after cisplatin & oxaliplatin Treatment

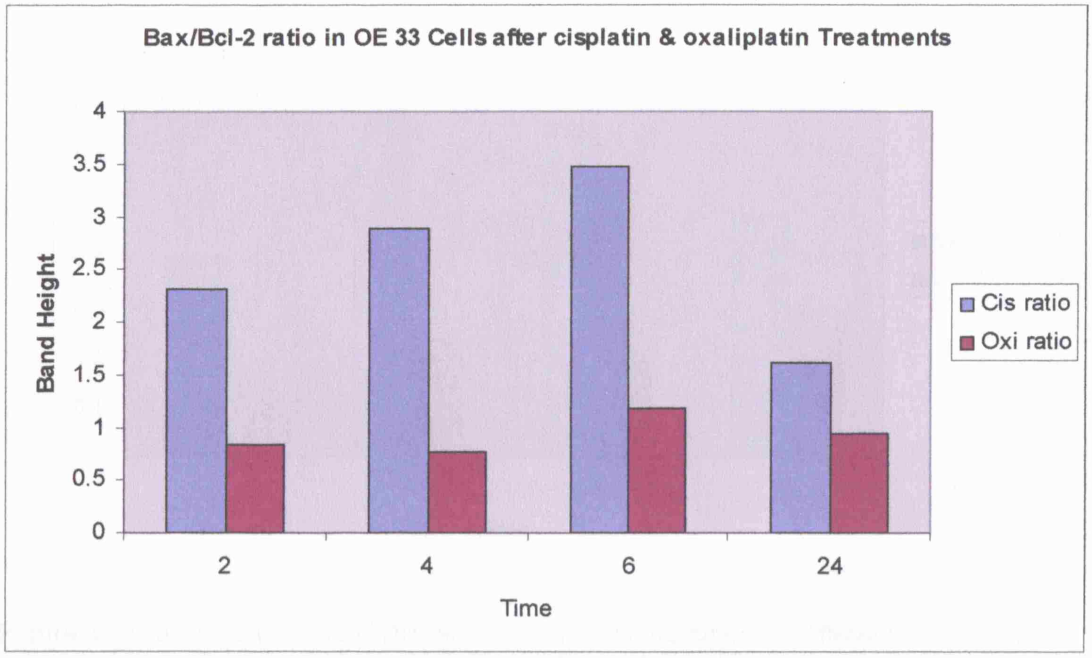


**Figure 33** The Bax/Bcl-2 ratio with cisplatin and oxaliplatin treatment at different time intervals calculated using Syngene® Gene Tools® and Microsoft Excel® programmes, which demonstrated that Bax was quite significantly expressed after cisplatin treatment at 2, 4 and 6 hours, while at 24 hours its expression was considerably low. However, Bcl-2 was expressed considerably less than Bax at all treatment intervals after oxaliplatin treatment

## Ratio of Bax & Bcl-2/GAPDH after cisplatin Treatment in OE 33 Cells

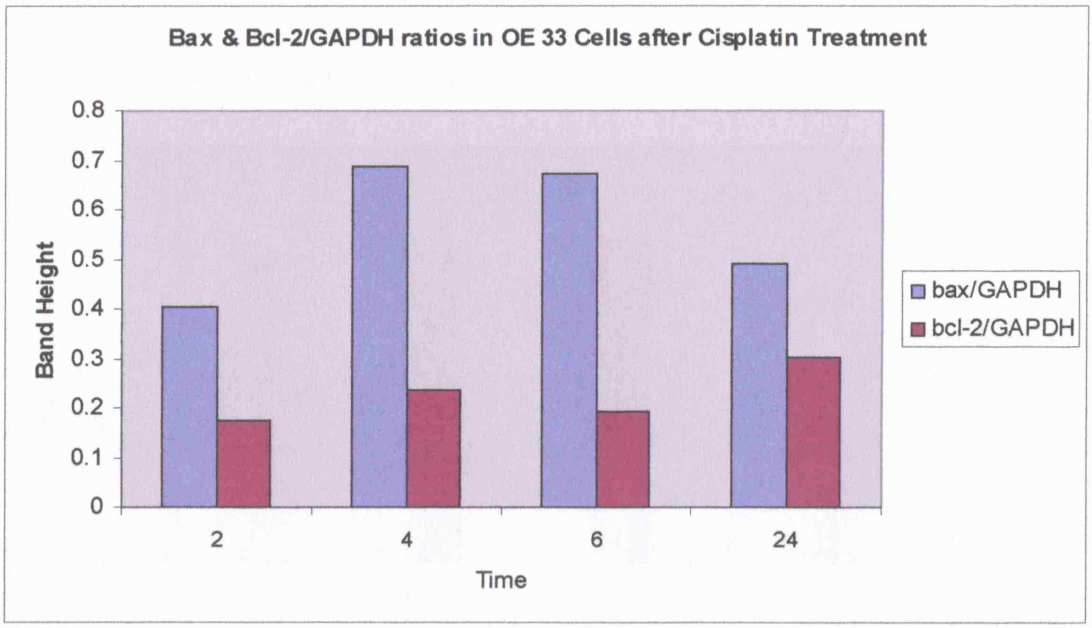


**Figure 34** The Bax & Bcl-2/GAPDH ratio after cisplatin treatment at different time intervals calculated using Syngene® Gene Tools® and Microsoft Excel® programmes, which demonstrated that Bax was quite significantly expressed in ratio to GAPDH at 2, 4, 6 and 24 hours, while Bcl-2 was significantly low in ratio to GAPDH at all treatment intervals

## Ratio of Bax & Bcl-2/GAPDH after oxaliplatin Treatment in OE 33 Cells

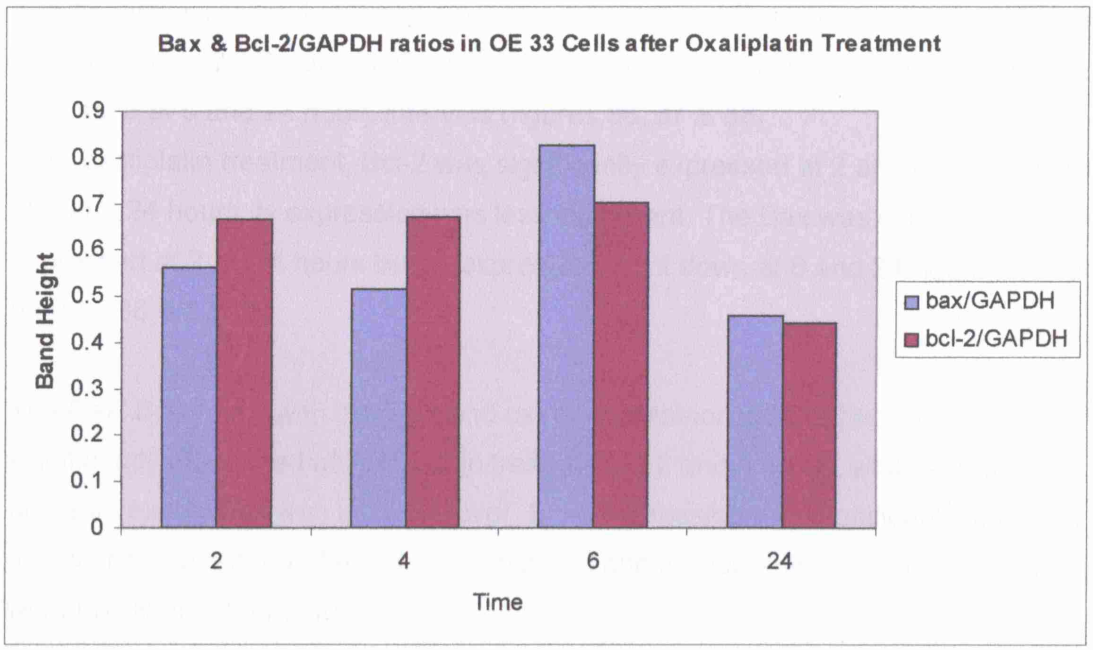


**Figure 35** The Bax & Bcl-2/GAPDH ratio after oxaliplatin treatment at different time intervals calculated using Syngene® Gene Tools® and Microsoft Excel® programmes, which demonstrated that Bcl-2 was significantly expressed in ratio to GAPDH at 2, 4, 6 and 24 hours. However, Bax was significantly low in ratio to GAPDH at 2, 4 and 24 hours while at 6 hours interval it was significantly expressed

## 6.4 Results of Bax and Bcl-2 expression in OE 21 cells

Band height analysis demonstrated that after cisplatin treatment, Bax was quite significantly expressed at 2 and 4 hours, while at 6 and 24 hours its expression was low. However, Bcl-2 was significantly low at 2 and 4 hours while its expression was increased at 6 and 24 hours intervals (figures 36, 37 & 38).
After oxaliplatin treatment, Bcl-2 was significantly expressed at 2 and 4 hours, while at 6 and 24 hours its expression was less significant. The Bax was significantly expressed at 2 and 4 hours but its expression went down at 6 and 24 hours intervals (figures 36, 37 & 39).

The Bax/ Bcl-2 ratio with cisplatin and oxaliplatin demonstrated that Bax was significantly expressed after cisplatin treatment at 2 and 4 hours, while at 6 and 24 hours its expression was low. However, Bcl-2 expression was significantly low after oxaliplatin treatment at 2 and 4 hours but at 6 and 24 hours intervals its expression went up slightly (figure 40).

The Bax & Bcl-2/GAPDH ratios demonstrated that Bax was significantly expressed after cisplatin treatment at 2 and 4 hours while at 6 and 24 hours its expression went down considerably. The expression of Bcl-2 was significantly low at 2 and 4 hours but increased significantly at 6 and 24 hours intervals (figure 41).
The Bax & Bcl-2/GAPDH ratios after oxaliplatin treatment demonstrated that Bcl-2 was significantly expressed at 2 and 4 hours while at 6 and 24 hours its expression went down. The Bax expression was significant at 2 and 4 hours but went down at 6 and 24 hours intervals (figure 42).

## Expression of Bcl-2 in OE 21 Cells after Cisplatin & Oxaliplatin Treatment

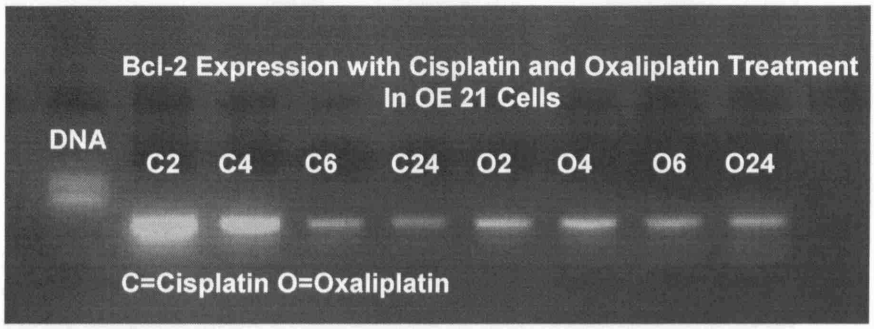


**Figure 36** Gene expression of bcl-2 (2% agarose gel) in OE21 cells following treatment with cisplatin and oxaliplatin at 2, 4, 6 or 24 hours intervals
C= Cisplatin, O= Oxaliplatin

## Expression of Bax in OE 21 Cells after Cisplatin & Oxaliplatin Treatment

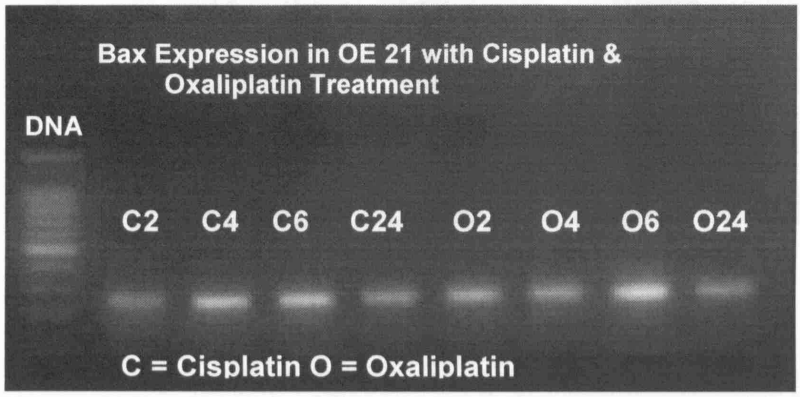


**Figure 37** Gene expression of Bax (2% agarose gel) in OE21 cells following treatment with cisplatin and oxaliplatin at 2, 4, 6 or 24 hours intervals
C= Cisplatin, O= Oxaliplatin

## Bax & Bcl-2 band heights in OE 21 Cells after Cisplatin Treatment

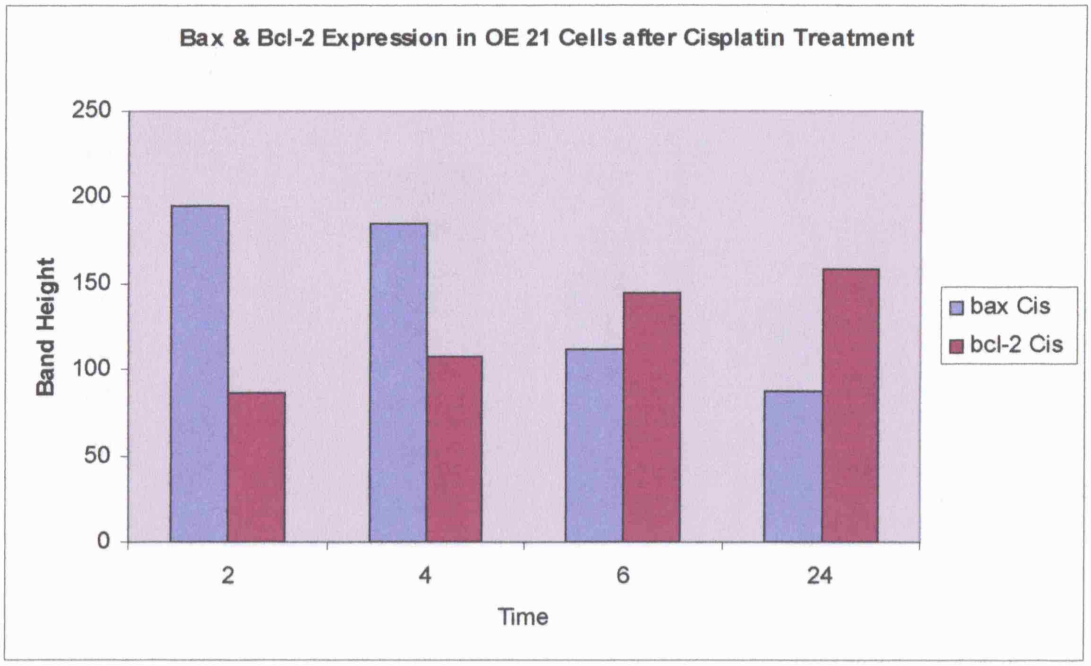


**Figure 38** Band heights of Bax and Bcl-2 after cisplatin treatment at different time intervals calculated using Syngene® Gene Tools® and Microsoft Excel® programmes, which demonstrated that Bax was quite significantly expressed after cisplatin treatment at 2 and 4 hours, while at 6 and 24 hours its expression was considerably low. However, Bcl-2 was significantly low at 2 and 4 hours while at its expression increased at 6 and 24 hours intervals.

## Bax & Bcl-2 band heights in OE 21 Cells after Oxaliplatin Treatment

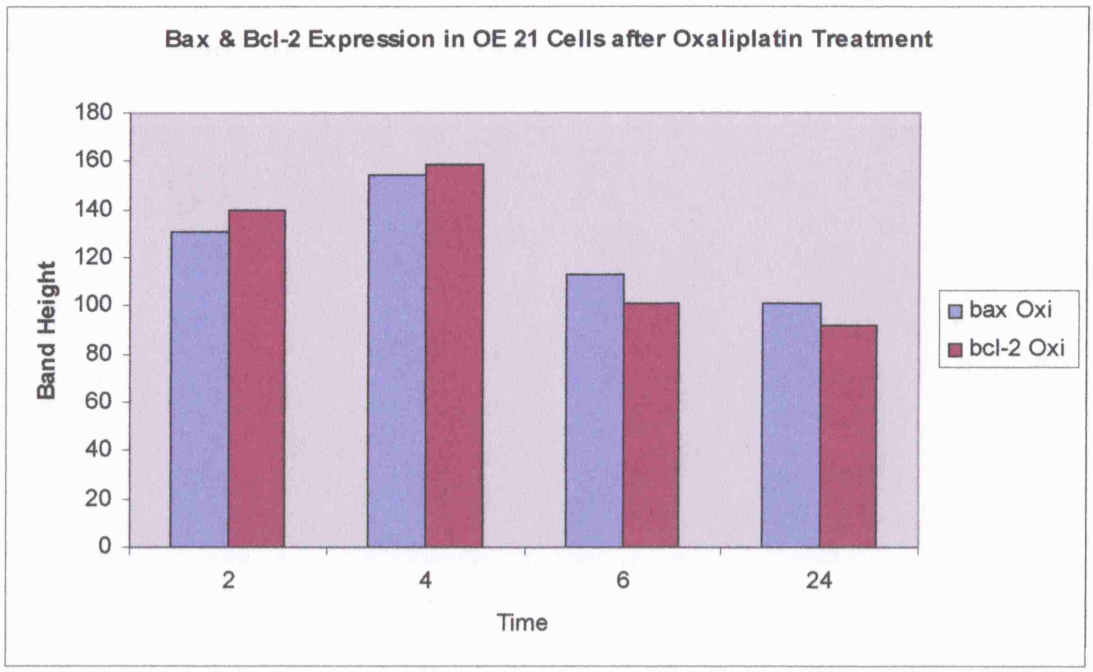


**Figure 39** Band heights of Bax and Bcl-2 with oxaliplatin treatment at different time intervals calculated using Syngene® Gene Tools® and Microsoft Excel® programmes, which demonstrated that Bcl-2 was significantly expressed after oxaliplatin treatment at 2 and 4 hours, while at 6 and 24 hours its expression was less significant. However, Bax was significantly expressed at 2 and 4 hours but its expression was decreased at 6 and 24 hours intervals.

## Bax/Bcl-2 Ratio in OE 21 Cells after Cisplatin & Oxaliplatin Treatment

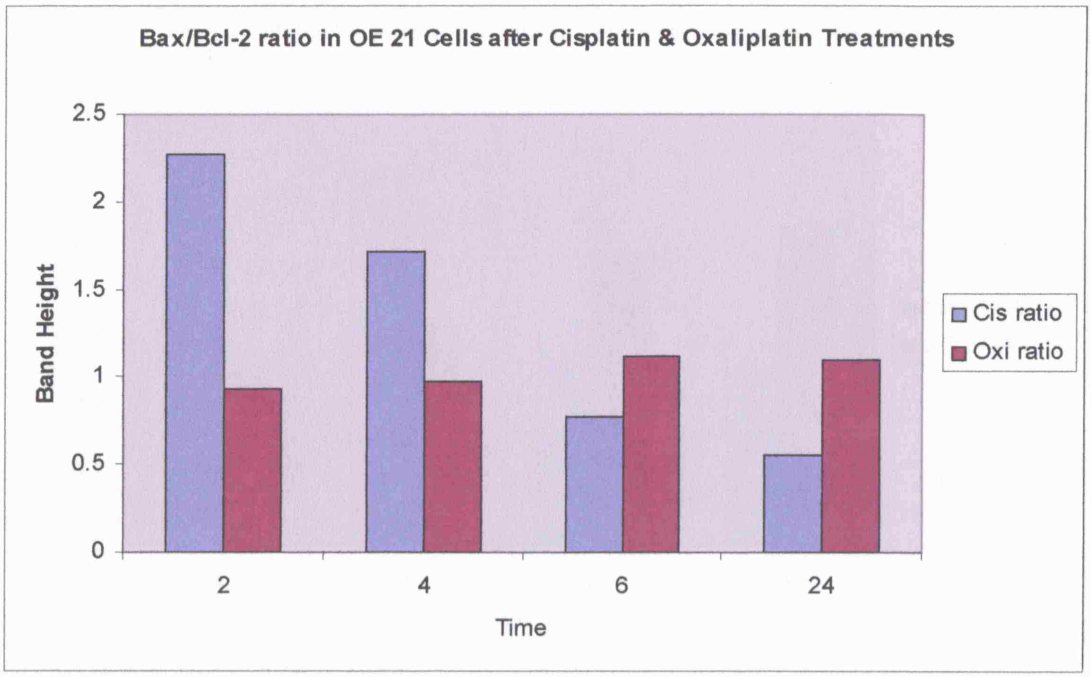


**Figure 40** Bax/ Bcl-2 ratio with cisplatin and oxaliplatin treatment at different time intervals calculated using Syngene® Gene Tools® and Microsoft Excel® programmes which demonstrated that Bax was quite significantly expressed after cisplatin treatment at 2 and 4 hours, while at 6 and 24 hours its expression was considerably low. However, Bcl-2 expression was significantly low after oxaliplatin treatment at 2 and 4 hours while at 6 and 24 hours intervals its expression went up slightly.

## Bax & Bcl-2/GAPDH Ratio after Cisplatin Treatment in OE 21 Cells

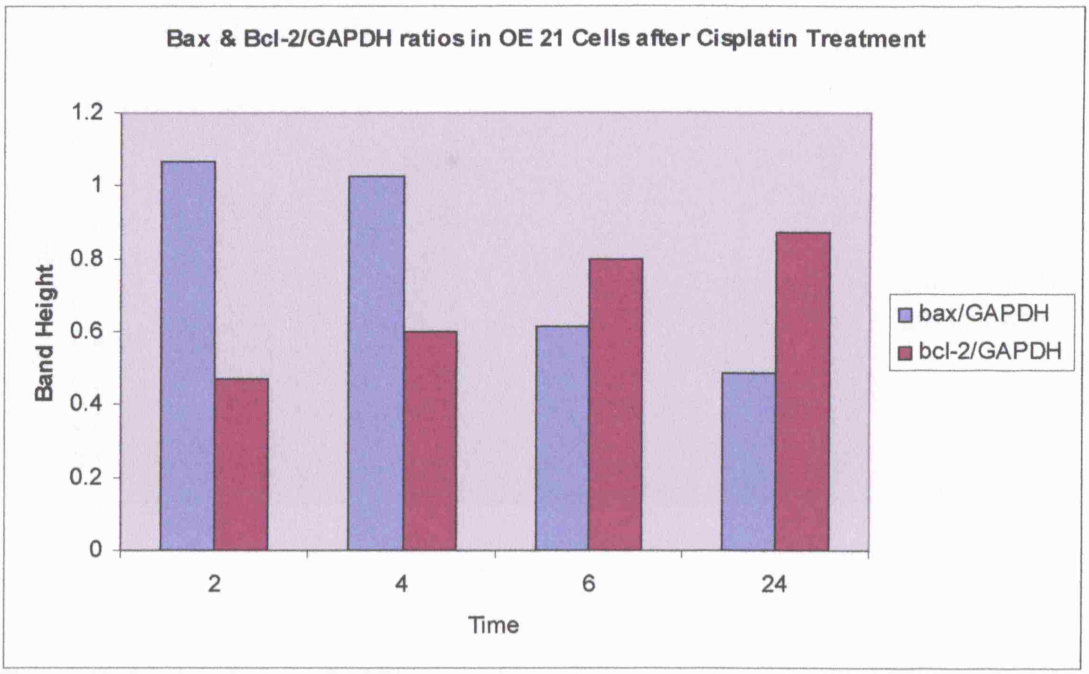


**Figure 41** Bax & Bcl-2/GAPDH ratio after cisplatin treatment at different time intervals calculated using Syngene® Gene Tools® and Microsoft Excel® programmes, which demonstrated that Bax was quite significantly expressed in ratio to GAPDH after cisplatin treatment at 2 and 4 while at 6 and 24 hours intervals its expression went down considerably. However, expression of Bcl-2 was significantly low in ratio to GAPDH at 2 and 4 hours but increased significantly at 6 and 24 hours intervals.

## Bax & Bcl-2/GAPDH Ratio after Oxaliplatin Treatment in OE 21 Cells

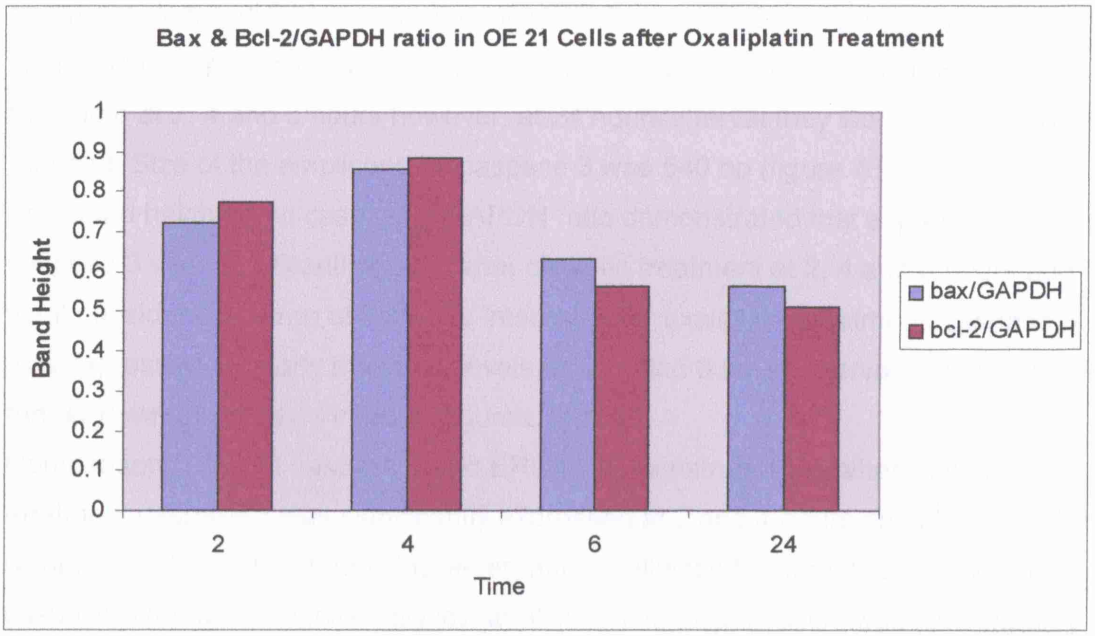


**Figure 42** The Bax & Bcl-2/GAPDH ratio after oxaliplatin treatment at different time intervals calculated using Syngene® Gene Tools® and Microsoft Excel® programmes, which demonstrated that Bcl-2 was significantly expressed in ratio to GAPDH at 2 and 4 while at 6 and 24 hours its expression went down considerably. However, Bax expression was significant at 2 and 4 hours but went down considerably at 6 and 24 hours intervals.

## 6.5 Results of Caspase 3 expression in OE 33 cells

Markedly elevated expression of caspase 3 was found after 2 and 4 hours of cisplatin treatment. At 6 and 24 hours treatment interval, caspase 3 expression was reduced significantly. After oxaliplatin treatment, levels of caspase 3 expression were nearly the same at 2, 4 and 6 hours however; at 24 hours interval they were considerably reduced. Size of the amplicons for caspase 3 was 540 bp (figure 43).

The Band heights and caspase 3/GAPDH ratio demonstrated that expression of caspase 3 was significantly higher after cisplatin treatment at 2, 4 and 6 hours and went considerably down at 24 hours interval. After oxaliplatin treatment, caspase 3 was expressed at nearly the same levels on 2, 4 and 6 hours intervals and went even further down at 24 hours interval (figures 44 & 45).

Comparison between caspase 3 and ERCC1 demonstrated that after cisplatin treatment caspase 3 was significantly expressed at 2 and 4 hours and its expression went down at 6 and 24 hours. However, after oxaliplatin treatment expression of caspase 3 remained significantly low at all time intervals. ERCC1 was considerably expressed at 6 hours interval after cisplatin treatment, while after oxaliplatin treatment it was very significantly expressed at 2, 4, 6 and 24 hours intervals (fig 46)

## Expression of Caspase 3 in OE 33 Cells after Cisplatin & Oxaliplatin Treatment

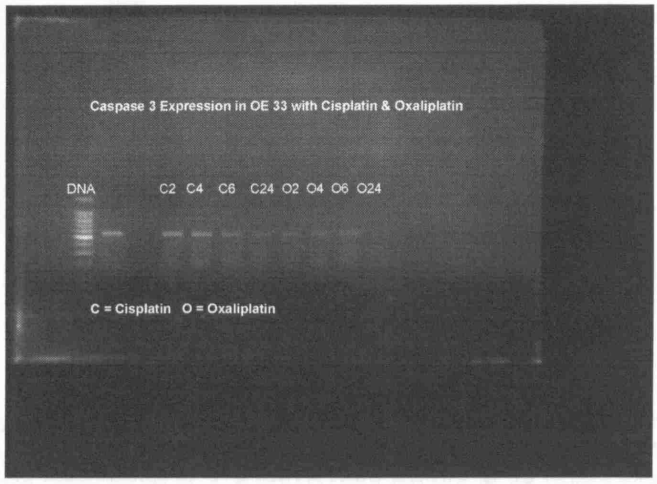


**Figure 43** Gene expression of Caspase 3 in OE33 cells following cisplatin and oxaliplatin treatment at 2, 4, 6 or 24 hours (2 % agarose gel)
C= cisplatin and O= oxaliplatin

## Caspase 3 band heights in OE 33 Cells after Cisplatin & Oxaliplatin Treatment

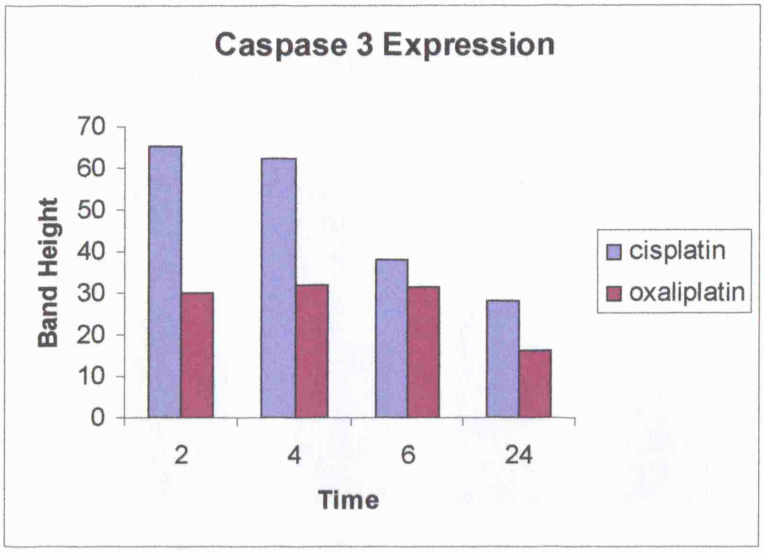


**Fig 44** Caspase 3 band heights for cisplatin and oxaliplatin calculated using Syngene® Gene Tools® and Microsoft Excel® programmes, which demonstrate that significant levels of caspase 3 were detectable at 2, 4 and 6 hour except at 24 hours where its levels were considerably low. After oxaliplatin treatment, levels of caspase 3 expression were nearly the same at 2, 4 and 6 hours however; they were quite low at 24 hours.

## Caspase 3/ GAPDH ratio in OE 33 cells after cisplatin and oxaliplatin Treatment

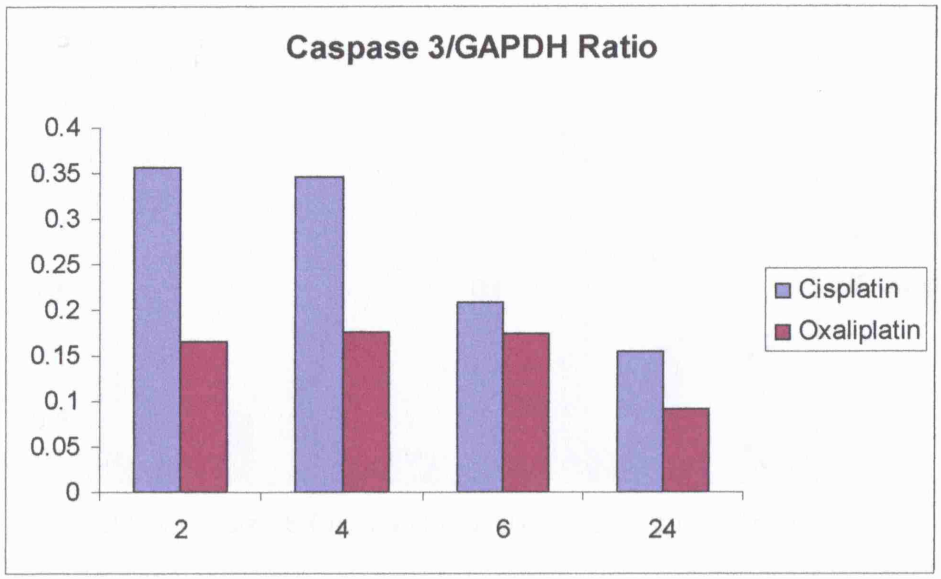


**Fig 45** Caspase 3/ GAPDH ratio for cisplatin and oxaliplatin calculated using Syngene® Gene Tools® and Microsoft Excel® programmes which demonstrate that significant levels of caspase 3 were detectable at 2, 4 and 6 hour except at 24 hours where its levels were considerably low. After oxaliplatin treatment, levels of caspase 3 expression were nearly the same at 2, 4 and 6 hours however; they were quite low at 24 hours

## Comparison between ERCC1 and Caspase 3 at Different Time Intervals after Cisplatin & Oxaliplatin Treatment in OE 33 Cells

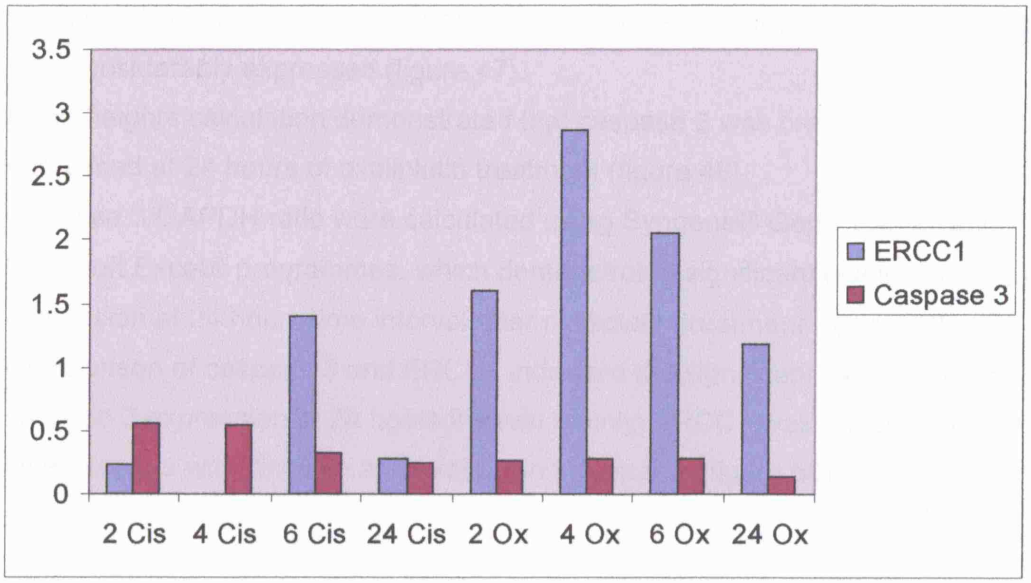


**Fig 46** Comparison between ERCC1 and caspase 3 at different time intervals calculated using Syngene® Gene Tools® and Microsoft Excel® programmes, which demonstrate that after cisplatin treatment caspase 3 was significantly expressed at 2 and 4 hours and its expression went slightly down at 6 and 24 hours. However, after oxaliplatin treatment caspase 3 expression remained significantly low at all time intervals. ERCC1 was considerably expressed at 6 hours interval after cisplatin treatment, while after oxaliplatin treatment it was very significantly expressed at 2, 4, 6 and 24 hours intervals.

## 6.6 Results of Caspase 3 expression in OE 21 cells

In the initial hours of cisplatin and oxaliplatin treatments caspase 3 was not significantly expressed, however after oxaliplatin treatment only at 24 hours interval it was considerably expressed (figure 47).
Band heights calculation demonstrated that caspase 3 was only significantly expressed at 24 hours of oxaliplatin treatment (figure 48).
Caspase 3/GAPDH ratio were calculated using Syngene® Gene Tools® and Microsoft Excel® programmes, which demonstrated significant levels of caspase 3 expression at 24 hours time interval after oxaliplatin treatment (figure 49).
Comparison of caspase 3 and ERCC1 indicated the significant expression of caspase 3 expression at 24 hours interval mainly; ERCC1 was undetectable at all time intervals with cisplatin and oxaliplatin treatments (figure 50).

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## Expression of Caspase 3 in OE 21 Cells after Cisplatin & Oxaliplatin Treatment

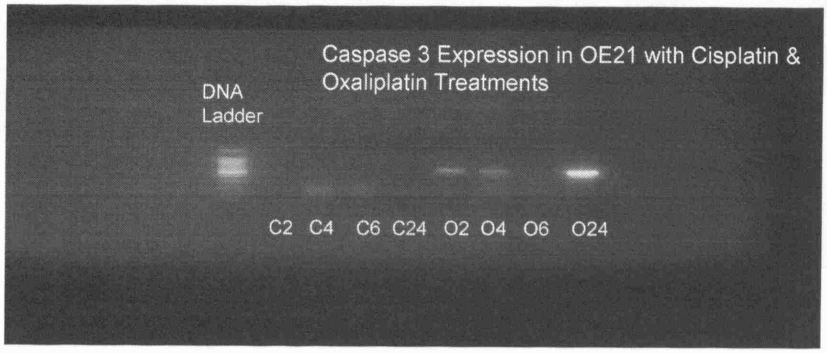



**Fig 47** Gene expression of Caspase 3 in OE 21 cells following cisplatin and oxaliplatin treatment at 2, 4, 6 or 24 hours (2 % agarose gel)
C= cisplatin, O= oxaliplatin

## Caspase 3 band heights in OE 21 Cells after Cisplatin & Oxaliplatin Treatment

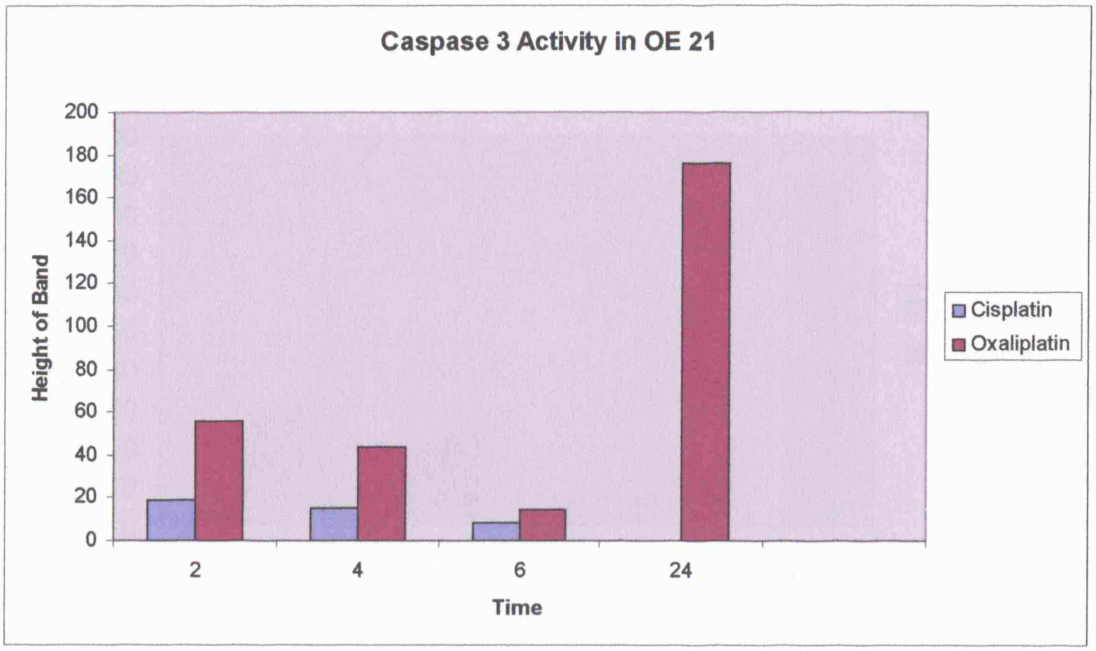



**Fig 48** The Band heights of caspase 3 at different time intervals calculated using Syngene® Gene Tools® and Microsoft Excel® programmes, which demonstrated that caspase 3 is significantly expressed at 24 hours interval.

## Caspase3/GAPDH Ratio in OE 21 cells after Cisplatin and Oxaliplatin Treatment

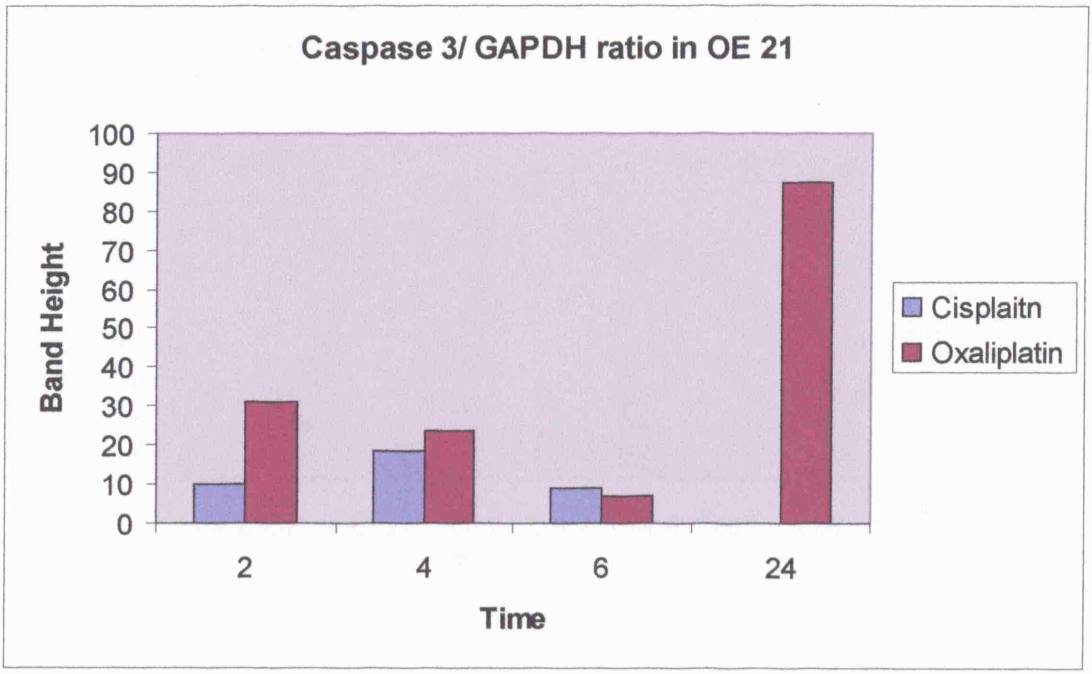



**Fig 49** Caspase 3/ GAPDH ratio in OE 21 cells after cisplatin and oxaliplatin treatments calculated using Syngene® Gene Tools® and Microsoft Excel® programmes which demonstrate that significant levels of caspase 3 were detectable at 24 hour after oxaliplatin treatment.

## Comparison between ERCC1 and Caspase 3 at Different Time Intervals after Cisplatin & Oxaliplatin Treatment in OE 21 Cells

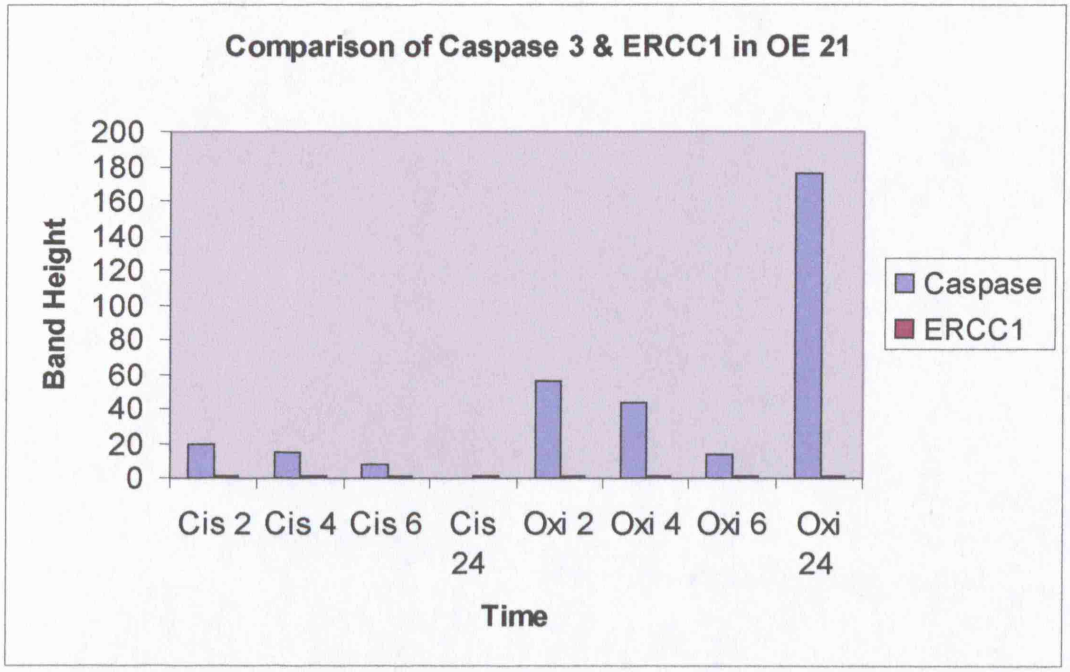



**Fig 50** Comparison between caspase 3 and ERCC1 at different time intervals calculated using Syngene® Gene Tools® and Microsoft Excel® programmes, which demonstrate that after cisplatin treatment caspase 3 was significantly expressed at 24 hours interval. ERCC1 was undetectable at all time intervals with cisplatin and oxaliplatin treatments.

# Chapter 7
## Discussion, Conclusion & Future Prospects

## 7.1 Discussion

Detail descriptions of the results are available in the relevant chapters however a brief summary is described here. In cytotoxicity experiments Lethal Dose $_{50}$ ($LD_{50}$) was calculated, oxaliplatin had greater $LD_{50}$ than cisplatin in both adenocarcinoma (OE33) and squamous cell carcinoma (OE21) cell lines. To confirm these findings Time Dependent Index (TDI) experiments were carried out and their results confirmed that cisplatin was more cytotoxic than oxaliplatin in OE33 cells. However in OE 21 cells both cisplatin and oxaliplatin had the same cytotoxicity level. To further evaluate the differences in their cytotoxicity, effects on ERCC1, XPA ,Bax, Bcl-2, caspase 3 gene expression and predictive value of ERCC1 were assessed.

In OE 33 cells after cisplatin treatment, band height of ERCC1 expression was nearly undetectable while after oxaliplatin treatment it was significantly expressed. On the other hand in OE 21 cells ERCC1 band heights were markedly expressed with both cisplatin and oxaliplatin treatment. In OE 33 cells XPA band heights were significantly expressed after oxaliplatin treatment, while with cisplatin treatment they were not detectable.

In OE 33 cells after cisplatin treatment band heights of Bax expression were significant in comparison with Bcl-2, which were expressed in considerably low amounts. While band heights of Bcl-2 were significantly expressed after oxaliplatin treatment than Bax band heights.

Bcl-2/Bax ratio was in favour of Bax in OE 33 cells after cisplatin treatment. However after oxaliplatin treatment the Bcl-2/Bax ratio was in favour of Bcl-2. On the other hand in OE 21 after cisplatin treatment the ratio of Bcl-2/Bax was in favour of Bax. Initially and in the late hours of treatment it turned in favour of Bcl-2. These results suggest that cisplatin was more cytotoxic in the initial treatment intervals than late ones. Expressions of Bax band heights were significantly high after oxaliplatin treatment and so did the expression of Bcl-2 band too. Thus the ratio of Bcl-2/Bax was not in favour of anyone of them which may suggests that oxaliplatin may be less cytotoxic in OE 21 cells.

In OE 33 cells expressions of caspase 3 band heights were significantly high after cisplatin treatment while after oxaliplatin treatment they were expressed in considerably low amounts. In OE 21 cells after cisplatin and oxaliplatin treatments caspase 3 band heights were not significantly expressed.

In UK, Epirubicin/Cisplatin/5FU (ECF) or Mitomycin C/Cisplatin/5FU (MCF) combinations are used for adenocarcinoma and Cisplatin/5FU (CF) combination is used for squamous cell carcinoma of oesophagus [26]. With the availability of new cytotoxic agents like oxaliplatin the challenge has been to show improved efficacy compared with the recognised standard. Therefore experiments were designed to compare the cisplatin and oxaliplatin in oesophageal cancers cells.

Cytotoxicities of cisplatin and oxaliplatin were evaluated by two different methods first by measuring the cytotoxicity levels using AlamarBlue™ assay and second exposing the two cell lines with cisplatin and oxaliplatin at different time intervals to assess the Time Dependent Index (TDI).

Arnould et al [160] used the $IC_{50}$ (the drug concentration that inhibit the 50% of cell growth) in colorectal cells for determining cytotoxicity of oxaliplatin and cisplatin. Similarly in the experiments of this project Lethal Dose 50 ($LD_{50}$) was calculated, which is the quantity of a drug needed to kill 50% of cells, the lesser the amount of a drug needed to kill 50% of the cells the more cytotoxic it would be or vice versa. Statistical analysis was performed using Prism 4® programme. It took the maximum and minimum values to calculate the $EC_{50}$ value, which represents the dose required to kill the 50% of the cells. This was in other words the mid point between the maximum and minimum where cell viability was 50%. In OE 33 cells the results demonstrated that only 30μM of cisplatin was needed in comparison to 79μM of oxaliplatin to kill 50% cells. The comparison of these two drug's $LD_{50}$ concentrations was carried out by calculating the p values that was 0.009, which is quite significant. These values suggested that cisplatin may be more effective than oxaliplatin in inducing apoptosis in OE 33 cells.
In OE 21 cells $LD_{50}$ dose of cisplatin was 25μM and for oxaliplatin it was 35μM, p values were calculated by comparing the $EC_{50}$ between cisplatin and oxaliplatin, it came as 0.14009, which is not significant. Thus, these observations suggested that in OE21 cell probably both drugs have the equal cytotoxicity level.

Analysis of TDI in OE 33 cells demonstrated that cisplatin was more cytotoxic than oxaliplatin at 2 and 4 hours interval. However at 6 hour the cytotoxicity ratio for both drugs was the same. It may be because the maximum numbers of cells were already dead by that time and there were not enough cells left to oxidise the AlamarBlue™. Correlations of these and $LD_{50}$ results demonstrated that cisplatin was more cytotoxic than oxaliplatin in OE33 cells.

In OE 21 cells, both cisplatin and oxaliplatin had the same cytotoxic ratio at 2, 4 and 6 hours which may suggests that at all time intervals both drugs had the same level of cytotoxicity. When these results were correlated with $LD_{50}$ results it was quite obvious that both drugs may have equal spectrum of cytotoxicity in these cells.

Arnould et al [160] found that the initial level of ERCC1 expression was correlated to cisplatin and oxaliplatin cytotoxicity; a high level of ERCC1 induced a greater removal of cisplatin and oxaliplatin DNA adducts and, consequently, a lower cytotoxic effect. Findings of ERCC1 expression in this project after cisplatin and oxaliplatin treatment were consistent with these observations. Results of ERCC1 expression exhibited on agorose gel calculated by Syngene® Gene Tools® demonstrated that it was nearly undetectable after cisplatin treatment in OE33 cells, while with oxaliplatin treatment it was significantly expressed. On the other hand in OE 21 cells ERCC1 was significantly expressed with both cisplatin and oxaliplatin treatment. These findings were further evaluated by measuring ERCC1/GAPDH ratios by using Syngene® Gene Tools® and Microsoft Excel® programmes. It may be suggested from these findings that oxaliplatin DNA adducts were effectively repaired, which made oxaliplatin less cytotoxic then cisplatin in OE 33 cells while in OE21 cells it may indicate that both cisplatin and oxaliplatin were not very effective in inducing cell death as their DNA adducts were effectively repaired.

Metzger et al [95] measured ERCC1 levels in gastric cancer patients treated with cisplatin/5-FU which demonstrated that the levels of ERCC1 influence the treatment outcomes. Shirota et al [219] have recently demonstrated the predictive value of ERCC1 expression in tumour samples from patients with colorectal cancer treated with oxaliplatin/5-FU. Median survival in patients with lower ERCC1 expression was better than with raised ERCC1 expression. Arnould et al [160] also showed the same results. These findings indicated that intratumoral ERCC1 may be used as an independent predictive marker for cisplatin or oxaliplatin chemotherapy. In OE33 cells after cisplatin treatment ERCC1 expression was low while after oxaliplatin it was high which may suggests decreased repair of DNA-adducts and more cells deaths and increased repair of DNA-adducts and decreased cell deaths respectively. These observations may suggest that ERCC1 can be used as a predictive marker in oesophageal cancer as well. These observations are also consistent with the findings of Britten et al [283] & Li et al [284] who demonstrated a correlation between ERCC1 expression level in tumours and the curative response of patients upon cisplatin chemotherapy.

XPA is involved in the very early steps of DNA damage recognition, associated with Replication Protein A. It plays a major role in NER since it was demonstrated that mutations on XPA prevents the interaction between XPA and ERCC1 and abolish DNA repair [285]. Arnould et al [160] also observed a correlation between XPA expression after oxaliplatin exposure and cytotoxicity. They found that when cells are able to keep XPA at a low level the residual level DNA adducts increases, inducing a higher cytotoxic effect. In OE 33 cells XPA was found significantly expressed after oxaliplatin treatment, while with cisplatin treatment it was not detectable measured on agorose gel by Syngene® Gene Tools®. These findings were further evaluated by measuring XPA/GAPDH ratios by using Syngene® Gene Tools® and Microsoft Excel® programmes. Correlation of ERCC1 and XPA results demonstrated that cisplatin-DNA adducts may not be repaired as effectively as oxaliplatin-DNA adducts, which made cisplatin more cytotoxic than oxaliplatin in OE 33 cells. These findings are consistent with other experiments of this project.

Dunkern et al [161] found that cisplatin-induced apoptosis in Chinese hamster ovary cells (CHO) appears to be regulated by changes in the Bax/Bcl-2 expression ratio, since they observed a decline of Bcl-2 protein level upon cisplatin sensitivity. Gourdier et al also [193] found that a high Bcl-2/Bax ratio favours cell survival, whereas a low ratio promotes apoptosis. In consistency with these findings Mese et al [270] found an increase in Bcl-2 amount in cisplatin resistant human epidermal carcinoma cells.
In OE 33 cells after cisplatin treatment the ratio of Bcl-2/Bax was in favour of Bax which suggests that cisplatin induced more cytotoxicity. However after oxaliplatin treatment in OE 33 cells the Bcl-2/Bax ratio was in favour of Bcl-2 which suggests that oxaliplatin was less cytotoxic in these cells.

On the other hand in OE 21 cells after cisplatin treatment the Bax was significantly expressed in the initial treatment intervals while at the late treatment intervals its expression went considerably down. The expression of Bcl-2 was significantly low in the initial treatment intervals while it increased significantly in the late treatment intervals. Thus the ratio of Bcl-2/Bax was in favour of Bax initially while it turned in favour of Bcl-2 in the late hours of treatment. These observations suggest that cisplatin was more cytotoxic in the initial treatment intervals while it was less toxic in the late treatment intervals. Although the expression of Bax was significantly high in the initial hours of oxaliplatin treatment but the expression of Bcl-2 was also high during those treatment intervals as well. While in the late hours of treatment

expression of both Bax and Bcl-2 went down. Thus the ratios of Bcl-2/Bax were same which may suggests that oxaliplatin may be less cytotoxic in OE 21 cells. These findings were first calculated from agorose gel readings by Syngene® Gene Tools® and then further evaluated by measuring Bax/GAPDH, Bcl-2/GAPDH ratios by using Syngene® Gene Tools® and Microsoft Excel® programmes.

Dunkern et al [161] analysed apoptotic signalling involved in the execution of cell death in NER defective cells. Their results demonstrated that low levels of ERCC1 display a high level of cisplatin-induced apoptosis, Bcl-2 decline and caspase activation which indicates that non-repaired cisplatin-induced DNA lesions act as trigger of the mitochondrial apoptotic pathway. They assumed from their experiments that non-repaired DNA breaks trigger the apoptotic pathway via Bcl-2 decline and caspase-9 and 3 activation. Azuma et al [286] discovered that cisplatin exerts its apoptotic action through the mitochondria-mediated activation of caspases (caspase-9 and caspase-3) in human oral squamous carcinoma (B88) cells.

Low levels of Bcl-2 expression were found in OE 33 cell after cisplatin treatment at all four time intervals. Interestingly when the expression of caspase 3 was assessed it was also found significantly high at all these four intervals. However after oxaliplatin treatment Bcl-2 was expressed significantly high at all four time intervals while caspase 3 was expressed in quite low levels. These findings were first calculated from agorose gel readings by Syngene® Gene Tools® and then further evaluated by measuring caspase 3/GAPDH ratio by using Syngene® Gene Tools® and Microsoft Excel® programmes.
When both these values were correlated with the findings of Azuma et al [ 286] & Dunkern et al [164] and with our Bax and ERCC1 values as well it was reasonable to suppose that cisplatin may used the non repaired DNA lesions to induce apoptosis via mitochondria more effectively than oxaliplatin. It may also reasonable to infer from these experiments that although oxaliplatin had used the mitochondrial apoptotic pathway but probably not with the same efficacy as cisplatin did in these cells.

Comparison of caspase 3 and ERCC1 expression values for both cisplatin and oxaliplatin gives a better understanding of their cytotoxicity in OE 33 cells. After cisplatin treatment, caspase 3 was expressed significantly, while ERCC1 was not, thus high caspase 3 and low ERCC1 levels indicated more apoptosis and less repair of cisplatin DNA adducts which may cause more apoptosis after cisplatin treatment. However after oxaliplatin treatment ERCC1 was significantly expressed while

expression of caspase 3 remained significantly low. High ERCC1 and low levels of caspase 3 may indicate more repair of oxaliplatin DNA adducts and less apoptosis, which made oxaliplatin less cytotoxic in OE 33 cells than cisplatin.

In OE 21 cells caspase 3 was not significantly expressed after cisplatin and oxaliplatin treatment. Comparison of caspase 3 and ERCC1 results may suggest that both these drugs were not very effective in inducing apoptosis or repairing platinum DNA adducts in OE 21 cells and may have approximately the same spectrum of cytotoxicity.

In summary the chain of events in the above mentioned results could be traced as following, DNA adducts of cisplatin were not effectively repaired by ERRC1/XPA which lead to the release of Bax and down regulation of Bcl-2 from mitochondria. The Bax promoted the formation of apoptosome complex that resulted in activation of caspase 3 which finally resulted in apoptosis in oesophageal cancer cells.
On the other hand the oxaliplatin DNA adducts were more effectively repaired by ERCC1/ XPA thus a less potent stimulus initiated the cytotoxic response. The findings of Bcl-2, Bax and Caspase 3 experiments demonstrated that this response was weaker in comparison to cisplatin. Thus it may be reasonable to infer from these experiments that cisplatin was more cytotoxic in comparison with oxaliplatin in OE 33 cells while cytotoxicity profile of both drugs was equal in OE 21 cells.

Above experiments have also demonstrated that ERCC1 may have a predictive value for cisplatin and oxaliplatin to chemotherapeutic response and resistance in OE 33 cells. The clinical value of such correlations in the prediction of cisplatin and oxaliplatin sensitivities could be tested in oesophageal cancer patients by determining ERCC1/XPA expression in tumour samples and comparing them with clinical outcome.

## 7.2 Conclusion

When the results of all experiments were correlated among themselves and with the findings of the other studies, it was may be reasonable to suppose that cisplatin has induced more apoptosis than oxaliplatin and it has used the mitochondrial apoptotic pathway more effectively than oxaliplatin in oesophageal cancer cells. Thus, in conclusion it may be fair to say that oxaliplatin in these cells has not shown the same good results as it did in colorectal cancers. Cisplatin is presently used in combination chemotherapy for oesophageal cancers. Results of the above experiments have

demonstrated that cisplatin was more effective than oxaliplatin in these cancers. Therefore replacing oxaliplatin with cisplatin may not be beneficial in oesophageal cancers.
Furthermore, expression of ERCC1 may have a predictive value for cisplatin and oxaliplatin chemotherapeutic response and resistance in oesophageal adenocarcinoma. However, further work using primary cells and prospective clinical trials may confirm these findings and their clinical benefit

### 7.3 Future Prospects

The concept that resistance to platinum drug, mediated by enhanced tolerance to adducts in DNA is rapidly gaining support. Several mammalian DNA polymerases capable of bypassing adduct that normally arrest replication has recently been identified [213, 215, 287]. Enhanced activity of such enzymes would be expected to permit survival under conditions of adduct load that would normally be lethal. Vaisman and colleagues [288] have recently reported that human DNA polymerases β, γ, and η can bypass oxaliplatin, as well as cisplatin adducts in DNA. Further experiments will be necessary to evaluate the exact role of polymerases in oxaliplatin cytotoxicity.

Oxaliplatin in the different cell lines never becomes associated with the DNA in the same amount. It is reasonable to expect that the efficiency of the mechanisms that mediate the delivery of oxaliplatin from the cytoplasm to the DNA might differ from tumour to tumour. This provides a conceptual basis, which could explain the relatively large difference, observed in the accumulation of different platinum drugs in the DNA [289]. It needs to be proved.

It will be quite beneficial to determine which Pt-DRP influence the cytotoxicity of DNA adducts, so that one can determine the carrier ligand specificity for binding to DNA adducts and evaluate the biological consequences of that binding. Information on carrier ligand specificity and biological effects of the proteins that interact with Pt-DNA adducts would be useful in the identification of molecular markers for predicting which platinum complexes will be most effective in treating individual tumours.

The resistance to oxaliplatin induced apoptosis strongly argues in favour of a defect in the mitochondrial apoptotic pathway [290]. Further studies are needed to investigate the ability of this drug to damage specific proteins, compared to the

cisplatin by the interaction of the hydrophobic DACH moiety of oxaliplatin with hydrophobic protein pockets in and outside mitochondria

Some cell lines that had been exposed to heavy metals expressed higher levels of metallothioneins and display cross-resistance to cisplatin [291]. Studies could be done on the interaction between metallothionein and oxaliplatin specificity.

# Appendices

## Appendix One
**Materials & Methods, Statistics and what is compared with what?**

### Serum Media Recommendations

The recommended media from ECACC for OE33 and OE 21 was Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% (v/v) Foetal Bovine Serum (FBS), 1 % (v/v) L-Glutamine. For prophylactic antibiotics, 1% (v/v) of streptomycin/penicillin was used. RPMI media was bought from Gibco Laboratories.

### AlamarBlue™ Assay

AlamarBlue™ assay was bought from serotec laboratories for the determination of Cytotoxacitiy of cisplatin and oxaliplatin.

### Reagents

Cisplatin and oxaliplatin were bought from the pharmacy of The Royal Free Hospital and stored at -20°C in 1 ml aliquots until used. All drug concentrations were made fresh on the day they were used.

### RT-PCR

Reverse transcriptase polymerase chain reactions (RT-PCR) were performed by using the One-Step RT-PCR Kit ® from QIAGEN ®.
Protocol for QIAGEN OneStep RT-PCR Kit and Q-Solution was used.

### Electrophoresis

PCR products were separated by electrophoresis on an agarose 2% gel and visualised by ethidium bromide staining using Syngene Gene Snap ® programme.

### Band Heights

Band heights for all PCR products were calculated using Syngene® Gene Tools® programme.

### Statistics

All the cytotoxicity assay data were analysed by using Prism Graph Pad Version 4 and Microsoft Excel® programmes. Log molar concentration of each drug was plotted against a common average cell viability value in Graph Pad Prism 4 programme to determine the lethal dose (LD) 50 for cisplatin and oxaliplatin. p value calculations were as follows.

## p value Calculation for Cytotoxicity assay OE 33

The curve fitting gave estimates of the EC50 (LD50) for cisplatin and oxaliplatin. To test the difference between EC50, we calculated the difference between log EC50 derived by a standard error for the differences in log EC50s and compared with a "t" distribution as follows

$$\text{Test Statistic} = \frac{\log EC50_{(Cis)} - \log EC50_{(Oxi)}}{\sqrt{(\text{Std. error of } \log EC50_{(Cis)})^2 + (\text{Std. error of } \log EC50_{(Oxi)})^2}}$$

Compare this with a "t" distribution

Degrees of freedom = $n_{(Cis)} + n_{(Oxi)} - 2$ x (numbers of parameters estimated for each curve)
Once the test statistics was calculated, p value can be obtained using Microsoft Excel® programme.
(Click "fx" on the tool bar, select statistical (from the category) then select TDIST click ok. Put in the "X" box the value calculated for "t" test, in the box degree of freedom put the combined values [e.g. in our case 12] and put "2" in the "Tails" box, this will give you the p value)

### Calculations for OE 33

$$\text{Test statistics} = \frac{(-4.521) - (-4.097)}{\sqrt{(0.08227)^2 + (0.1076)^2}}$$

$$= -0.424/0.1317 = 3.12$$

Degrees of freedom = 12
p values calculated using Microsoft Excel® programme as explained above
p= 0.009

### Calculations for OE 21

$$t = \frac{(-4.566)-(-3.488)}{\sqrt{0.1895^2 + 0.1498^2}}$$

= -1.078

0.6786

= -1.58

Degrees of freedom = cisplatin + oxaliplatin

6+6 = 12

p values calculated using Microsoft Excel® programme as explained above

p = 0.14009

## Confidence Interval

### Cisplatin

| Conc | 0 | 0.001 | 0.0005 | 0.0001 | 0.00005 | 0.00001 | 0.000005 | 0.000001 |
|---|---|---|---|---|---|---|---|---|
| Mean | 223.13 | 129.78 | 142.025 | 146.485 | 163.92 | 194.35 | 205.925 | 214.375 |
| Confidence interval (95%) upper | 227.3749 | 136.7395 | 147.3621 | 151.8397 | 167.4832 | 209.6359 | 214.8219 | 223.1896 |
| Confidence interval (95%) lower | 218.8851 | 122.8205 | 136.6879 | 141.1303 | 160.3568 | 179.0641 | 197.0281 | 205.5604 |

| 5E-07 | 1E-07 |
|---|---|
| 213.905 | 211.28 |
| 221.4708 | 218.6889 |
| 206.3392 | 203.8711 |

### Oxaliplatin

| Conc | 0 | 0.001 | 0.0005 | 0.0001 | 0.00005 | 0.00001 | 0.000005 | 0.000001 |
|---|---|---|---|---|---|---|---|---|
| Mean | 225.275 | 125.675 | 125.7 | 155.53 | 176.92 | 196.29 | 200.345 | 209.21 |
| Confidence interval (95%) upper | 229.6268 | 132.546 | 130.1814 | 166.1702 | 187.9761 | 210.7164 | 210.8652 | 218.1725 |
| Confidence interval (95%) lower | 220.9232 | 118.804 | 121.2186 | 144.8898 | 165.8639 | 181.8636 | 189.8248 | 200.2475 |

| 0.0000005 | 0.0000001 |
|---|---|
| 210.995 | 211.885 |
| 219.85084 | 218.56043 |
| 202.13916 | 205.20957 |

The values for the confidence intervals are calculated on the untransformed data. This means that the means shown here may be different from those seen on the graphs.

### Histograms calculated from band height

These data represent standardised band heights. The height of a reporter gene (GAPDH) was used to create a ratio with the gene being studied. This means that the transformed data is not suitable for confidence limits.

### What is compared with what and why used the word 'significantly?

RT-PCR produce genes were measured on agorose gel by Syngene® Gene Tools® for all genes expression experiments. The heights of the bands for different genes were calculated using Syngene® Gene Tools® produced numbers and if they were expressed quite clearly in comparison with GAPDH, the word significantly was used to describe their importance.
GAPDH is referred as a 'house-keeping' protein since its expression remains relatively abundant and constant under changing cellular conditions and it acts as an internal control for the PCR reaction in most cells. Therefore all RT-PCR genes expressions were correlated with GAPDH levels and compared with each others. For example as in Metzger et al[Ref?] study ERCC1 levels, measured by quantitative PCR in gastric cancer patients treated with cisplatin / FU. cDNA was obtained from primary gastric tumours before chemotherapy, and ERCC1 levels were expressed as the ratio of the PCR product of the ERCC1 gene and the β-actin housekeeping gene.

### Graph Pad® version 4 and Microsoft Excel® Programmes for Calculation of LD 50

Cytotoxicity values obtained, either from Multiskan Lab system, (which reads colorimetric absorbance of alamarBlue assay) or fromThermo Lab system (that takes fluorometric plate readings with Ascent Software version 2.6) are inserted into Excel®. First, an average is calculated for all values separately. Then each average is divided by the control average value individually. Multiply each value with 100. This will give the final reading for each well separately, as each value represent a well with a specific drug concentration. Insert these values against any specific drug concentration used, in Graph Pad Prism and it will calculate the LD 50.

## Appendix 2
## Determination of Platting Densities

## Aims

Two variables affect the response of cells to AlamarBlue™, are the length of incubation time and the number of cells plated. Thus, it was important to know the correct plating densities for OE 33 & OE 21 cell lines.

## Materials & Methods

OE 33 and OE21Cells were platted in 24 well plates in different densities ranging from 2 x $10^3$ to 5 x $10^5$ cells/ml. AlamarBlue™ assay was used to measure the cell viability. Multiskan Lab system was used for colorimetric plate readings and assessing the AlamarBlue™ absorbance monitored at 570nm and 600nm wavelength. Thermo Lab system was used for fluorometric plate readings monitored at 560nm excitation wavelength and 590nm emission wavelength using Ascent Software version 2.6 for assessing AlamarBlue ™ excitation and emission.

## Results

In the fluorometric plate readings results (figure 69), the metabolism of AlamarBlue™ started quickly at cell concentration 5x$10^5$ and reached to its peak in around less than three hours. At 2x$10^5$ metabolism of AlamarBlue™ started quickly again but attained its peak less slowly than the previous cell concentration i.e. in approximately eight hours. At 1x$10^5$ cell concentration AlamarBlue™ metabolism started gradually, reached its peak in smoother manner and attained its peak in approximately eighteen hours. While in the rest of the cell concentrations, every cell concentration attained its peak very slowly. In colorimetric plate reading results (figure 70), at 5x$10^5$cell concentration metabolism of AlamarBlue™ started quickly and reached its peak in around less than three hours, while at 2x$10^5$ cell concentration metabolism of AlamarBlue™ started quickly but attained its peak less slowly. At the cell concentration of 1x$10^5$ AlamarBlue™ metabolisms started after a while and gradually reached its peak and attained its peak in approximately eight hours. Rest of the cell concentrations attained their peak very slowly

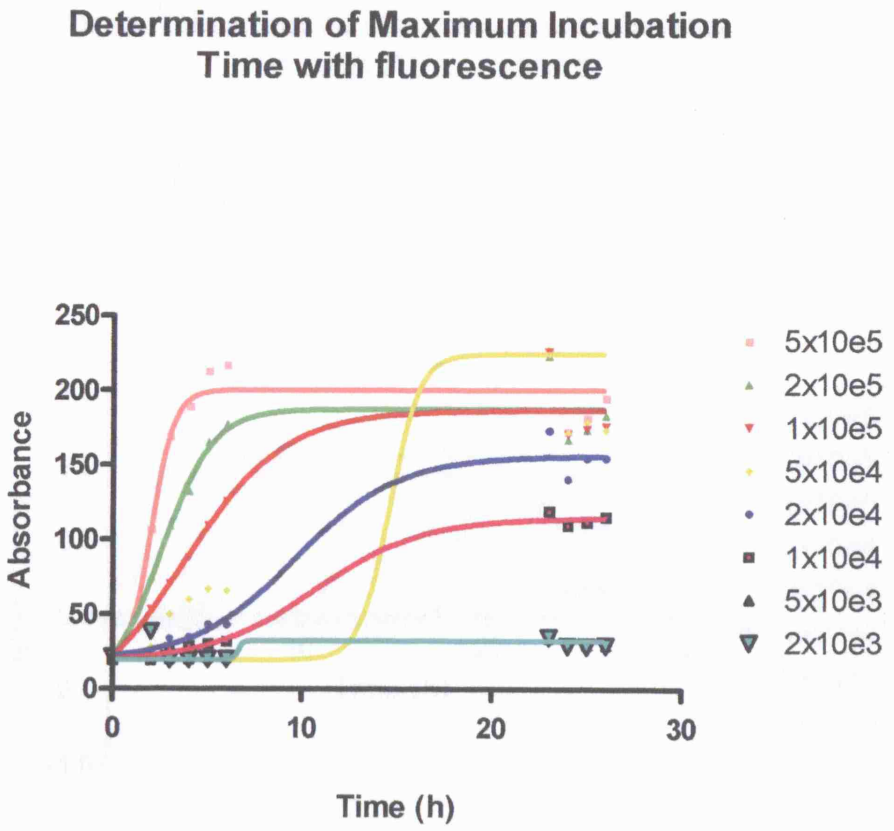



**Figure 51** Determination of maximum incubation time using AlamarBlue™ absorbance for different number of cell densities. Thermo Lab system was used for fluorometric plate readings with Ascent Software version 2.6. Results are the mean of three independent experiments.
At $5x10^5$cell concentration metabolism of AlamarBlue™ started quite quickly and reached its peak in around less than three hours. At $2x10^5$ metabolism of AlamarBlue™ started quite quickly again but attained its peak less slowly than the previous cell concentration in approximately eight hours. At the cell concentration $1x10^5$ AlamarBlue™ metabolisms started quite gradually, reached its peak in smoother manner and attained its peak in approximately eighteen hours than the previous two concentrations where it reached its peak quite steeply. Rest of the cell concentrations attained their peak quite less slowly as is shown in the graph.

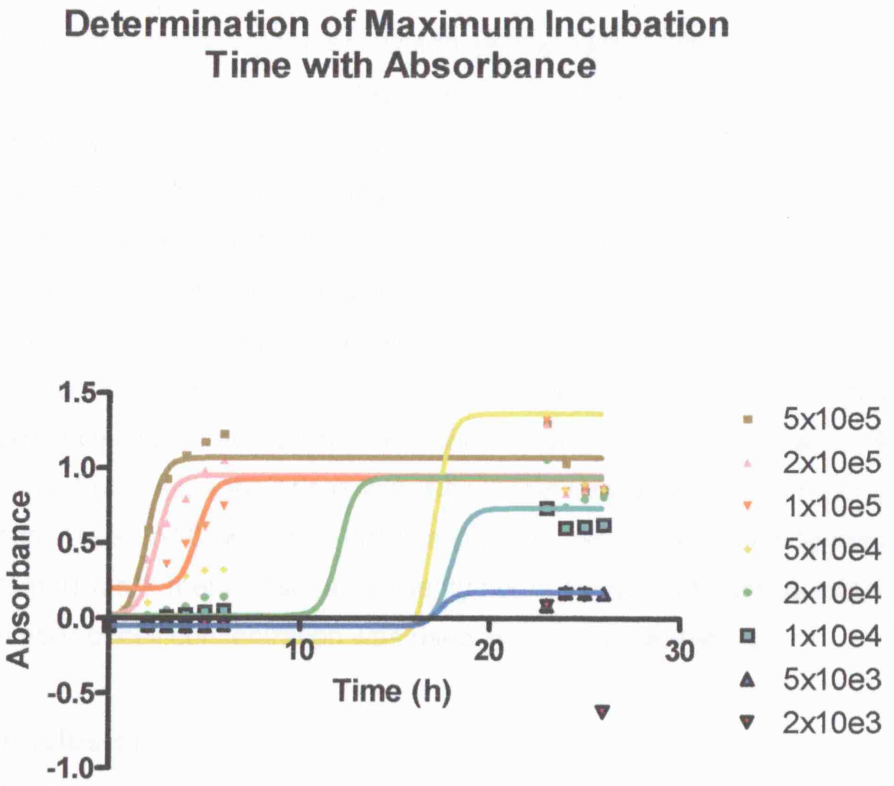



**Figure 52** Determination of maximum incubation time using AlamarBlue™ absorbance for different number of cell densities. Multiskan Lab system was used for colorimetric plate readings. Results are the mean of three independent experiments.

At $5x10^5$cell concentration metabolism of AlamarBlue™ started quite quickly and reached its peak in around less than three hours. At $2x10^5$ metabolism of AlamarBlue™ started quite quickly but attained its peak less slowly than the previous cell concentration in approximately five hours. At the cell concentration $1x10^5$ AlamarBlue™ metabolisms started after a while and quite gradually, reached its peak in smoother manner and attained its peak in approximately eight hours, in comparison of the previous two cell concentrations where it reached its peak quite steeply. Rest of the cell concentrations attained their peak quite less slowly as is shown in the graph.

## Discussion

The reason of this experiment was to get the right number of cell concentration that can give the correct results of AlamarBlue™ metabolism after overnight incubation. Secondly, to assess the results of these two different systems of plate readings in order to cheek the authenticity of each others results.

In the fluorometric and colorimetric results although few differences were noted in the pattern of AlamarBlue™ metabolism with the cell concentration ranging from $5x10^5$ to $1x10^5$, which are explained above in the result section but these differences are probably interpretational differences of the results by each system, otherwise the correct cell concentration for overnight incubation remains the same. Rest of the cell concentrations got their peak very slowly that may given the erroneous results if used. Thus, $1x10^5$ was the correct number of cell concentration to read the results of AlamarBlue™ metabolism after twenty hours or overnight period and the same number of cell concentration was used in above experiments.

## Conclusion

Above experiments have suggested that for OE 33 and OE 21cell lines $5x10^5$ was the right cell concentration for plating and incubation

## Appendix Three
## Lab Procedures

## Procedure for Handling Frozen Cell Ampoules on Arrival

Cell were arrived in 1ml plastic cryotubes containing cells and FBS 10% (v/v) dimethylsuiphoxide (DMSO). The ampoule was packed in solid carbon dioxide ($CO_2$) pellets. Ampoule was transferred to the liquid nitrogen immediately after arrival.

### Resuscitation of Frozen Cells

The ampoule was taken out from liquid nitrogen and kept at room temperature for approximately 1 minute before immersing it in waterbath at 37°C for 1-2 minutes so that cells can fully defrosted. Hold the ampoule in hand while thawing otherwise it will submersion in water and will be contaminated. Ampoule was wiped with a tissue soaked in 70% alcohol prior to opening.

The whole contents of the ampoule were slowly pipetted into a 25ml flask containing pre warmed 10 ml of RPMI 1640 media (containing 10% (v/v) FBS,1 % (v/v) L-Glutamine and 1% (v/v) of streptomycin/penicillin). Cells were incubated at 37°C in a humidified atmosphere containing 5% $CO_2$. Cells were left to grow for 24-48 hours until they were 70-80% confluent and ready for splitting.

### Splitting of Growing Cells

When the growing cells were 70-80% confluent they were splitted and transferred into a bigger 100ml flask. The 25ml flask in which cells were resuscitated from the frozen ampoule was washed with phosphate Buffer Solution (PBS) 10ml x three times. Two ml of 0.25% trypsin/EDTA was used to harvest the cells from the flask surface; flask was left in the incubator for 4-5 minutes at 37°C in a humidified atmosphere containing 5% $CO_2$. When cells were completely floating in the flask, which was confirmed by looking under the microscope, 10ml of complete RPMI 1640 was added to the flask to neutralize the trypsin. Contents of the flask were centrifuged at the speed of 600g for 5 minutes after transferring them in a 20ml universal. The media from the universal was discarded and the cells were suspended into fresh 10ml of complete media. Cells were dispersed by pipetting. Five 100 ml flasks were used for running culture, in each of which, 13 ml of fresh complete media and two ml of the cells were added. Flasks were left to grow at 37°C in a humidified atmosphere containing 5% $CO_2$.

### Making Complete Media

The recommended media from ECACC for our OE33 cells is Roswell Park Memorial Institute (RPMI)1640 supplemented with 10% (v/v) Foetal Bovine Serum (FBS),1%(v/v) L-Glutamine and 1% (v/v) of streptomycin/penicillin.
In the bottle of 500ml RPMI media, we added 50 ml of FBS, which represent 10% (v/v), 5ml of L-Glutamine which is 1% (v/v) and for prophylaxis 5ml of streptomycin/penicillin solution, which is 1%(v/v).

### Making Serum Negative Media

When the cells were treated with cytotoxic drugs, drugs were dissolved in serum negative media instead of serum positive. Making of this media is same as serum positive media with the exclusion of FBS.

### Maintaining Growing Cells

Media of the growing cells was changed in 3-4 days and when the cells were 70-80% confluent in nearly a week's time, they were split as per protocol. Sixteen 100 ml flasks of cells were grown before freezing the cells for future use.

### Procedure for Freezing Cells

When the sixteen 100ml flasks were nearly 80-90% confluent with cells, twelve flasks were freezed for future use and back up.
The cells were splitted in the same manner as described earlier on just with one exception, which is, instead of adding media for resuspension, cells were resuspended in one ml of 90% serum (FBS) and 10% dimethylsuiphoxide (DMSO), cryoprotectant solution. Cells with cryoprotectant solution were pipetted into clearly marked one ml ampoules. These ampoules were transferred into -80°C freezer immediately. On the next day, cells were taken out from the freezer (-80 °C) and transferred into the liquid nitrogen.

### Different ways of counting cells by using Haemocytometer

After splitting the cells as described previously, they were resuspended into two mls of media instead of 10mls and thoroughly dispersed using one ml Gilson pipette. Later on 8ml of media was added to make it 10 ml.
For cell counting, in 100µl of cell solution, 100µl of Trypan Blue was added to make up a solution. Alternatively, a solution can be made by taking 500µl of cell solution + 400µl of cold PBS and 100µl of Trypan Blue.

Cells were counted after putting a tiny drop over haemocytometer from either of the above two solution.

Haemocytometer is made up of nine big squares, the central square is further subdivided into 16 small squares and each of these small squares is further subdivided into 16 tiny squares. Cells on haemocytometer were counted by two methods

### First Method of counting cells on Haemocytometer

Cells were counted in the central square and following formula was used.

Number of cells $1cm^3 \times 10^4 \times$ dilution factor = Total number of cells/ml

Dilution factor was 2 as Trypan blue was used.

For example

Number of cells was 85

$(85)(10^4) \times 2 = 850000$ cells per ml in 10mls universal container

### Second Method

Cells can also be counted in the central and in any other square and both squares were added together, in that case, there was no need to multiply the total number of cells with dilution factor. Numbers of the cells were roughly the same in all of nine the squares of haemocytometer.

### Making Different cell concentrations

Different cell concentrations were needed for different purposes so following methods were used for making different cell concentrations.

For example, total number of the cells is $134x10^4$ /ml and, however the correct concentration sought was $5x10^5$ per ml in 80mls,

80mls @ $5x10^5$

$= 80x5x10^5 = 400x10^5 = 4x10^7$ (Total number of cell in 80mls)

Cells we got $134x10^4$,

$= 4x10^7 / 134x10^4 = 4x10^3/134 = 4000/134 = 29.85$ mls of cell (from 10 mls Cell solution)

29.85 cells solution + 50.15 of media = 80 mls , that was the concentration used for getting 80mls of the solution @ $5x10^5$ of cells/ ml.

To make sure that our above reading was correct reverse calculations were performed as follows.

Total number of cells in 80 mls @$5x10^5$/ml = $4x10^7$ =40000000

29.85 x $134x10^4$ = 3999.99 x $10^4$

= 4000 x $10^4$ = $4x10^7$

= 40000000, hence it proved that our calculations were correct

## Platting Cells in 24 Wells Plate

Cells were split and counted as described above. On different occasions, different cell concentrations were used, so one example is given here here to show how the number of cells was counted in each well for 24 wells plate.

If $1x10^5$ numbers of cells per ml for 24 wells plate were needed and the total number of cells for example was $134x10^4$

For 24 wells plate

= 24x $1x10^5$ = 24 x $10^5$ =2400000 (total number of cell)

Provided number of cells $134x10^4$ cells/ ml

Number of cells Needed

24 x $10^5$,

24 x $10^5$ / $134x10^4$ = 240/134 = 1.79 or 1.8 mls (approx)

1.8mls of cells + 22.2mls of media will give $1x10^5$ number of cell per ml for 24 wells plate.

## Reverse Calculation

1.7910 x $134x10^4$ = 240 x $10^4$ = 2400000 number of cells , proved.

Another way of doing the above calculations can be

Quantity of cells needed

$1x10^5$ /ml

Number of cells provided

$134x10^4$ cells/ ml

$134x10^4$ / $1x10^5$ = 134/10 = 13.4

Out of 13.4, 1ml of cell suspension and 12.4mls of media will provide $1x10^5$ / number of cells per ml.

For 24 wells

Cell ---------- Media

Solution

1ml ---------- 12.4mls, we need 24 mls @ $1x10^5$ /ml so,

1x1.8----------12.4 x1.8

= 1.8---------- = 22.32

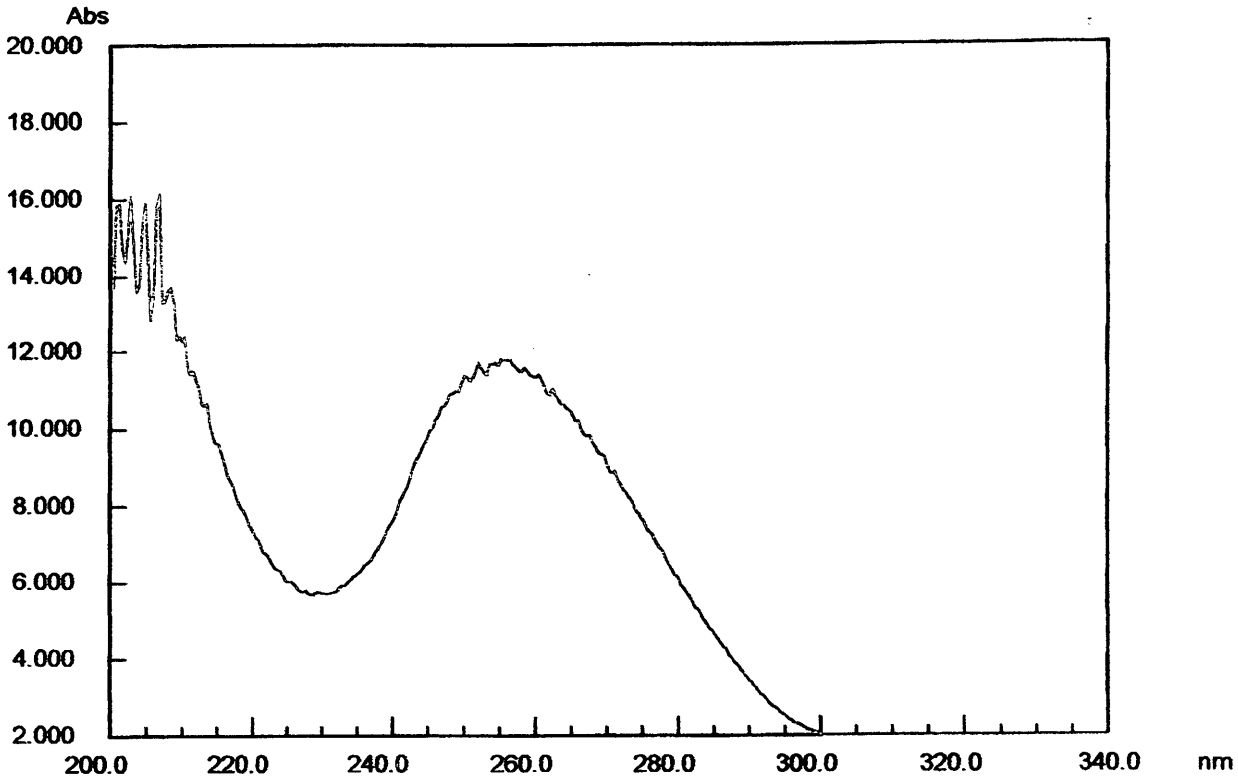
1.8+ 22.32 = 24.12

Or

Approximately, two mls of cell solution + 24.8 ml of media = 26.8, can provide the same concentration for 24 wells plate, rest of it can be discarded.

**Appendix Four**
**Gene Spec RNA Graphs**

Report: 04/8/17 12:25




Result

| 230.0 (nm) | 260.0 (nm) | 280.0 (nm) | Ratio | Conc (ug/uL) | Pure (%) | Flag |
|---|---|---|---|---|---|---|
| 5.730 | 11.315 | 6.133 | 1.845 | 0.45 | 92.2 | |

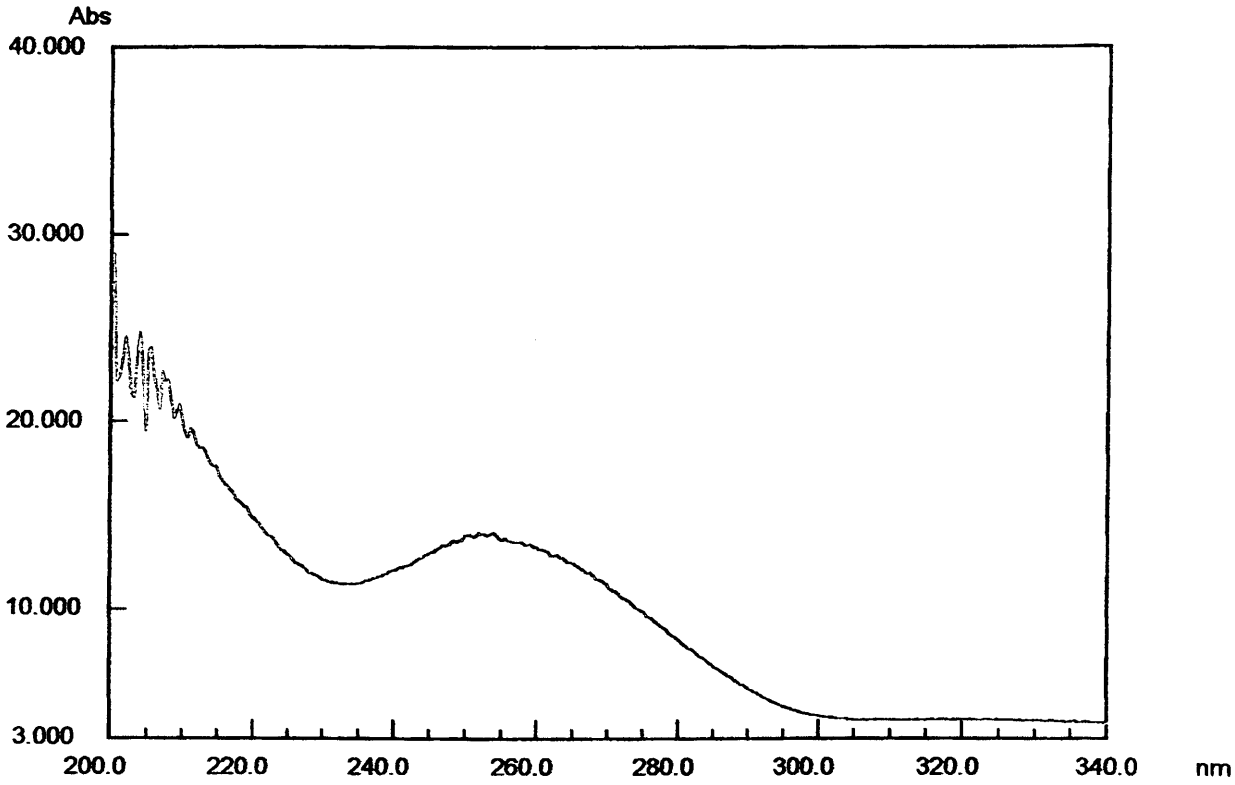
Date : 04/8/17 12:11
Sample :
Comment :

Instrument
Integration : 32

DNA Parameter
Mode : RNA
Dilution : 1.00
Background : Off
Factor : 40.0
Pure : ON
P.Conc : Off
M.Conc : Off
Unit : ug/uL

Upper : 100.0
Lower : 0.0
Expeted Value : 2.000

Report: 04/8/16 13:28




Result

| 230.0 (nm) | 260.0 (nm) | 280.0 (nm) | Ratio | Conc (ug/uL) | Pure (%) | Flag |
|---|---|---|---|---|---|---|
| 11.573 | 13.302 | 8.323 | 1.598 | 0.53 | 79.9 | |

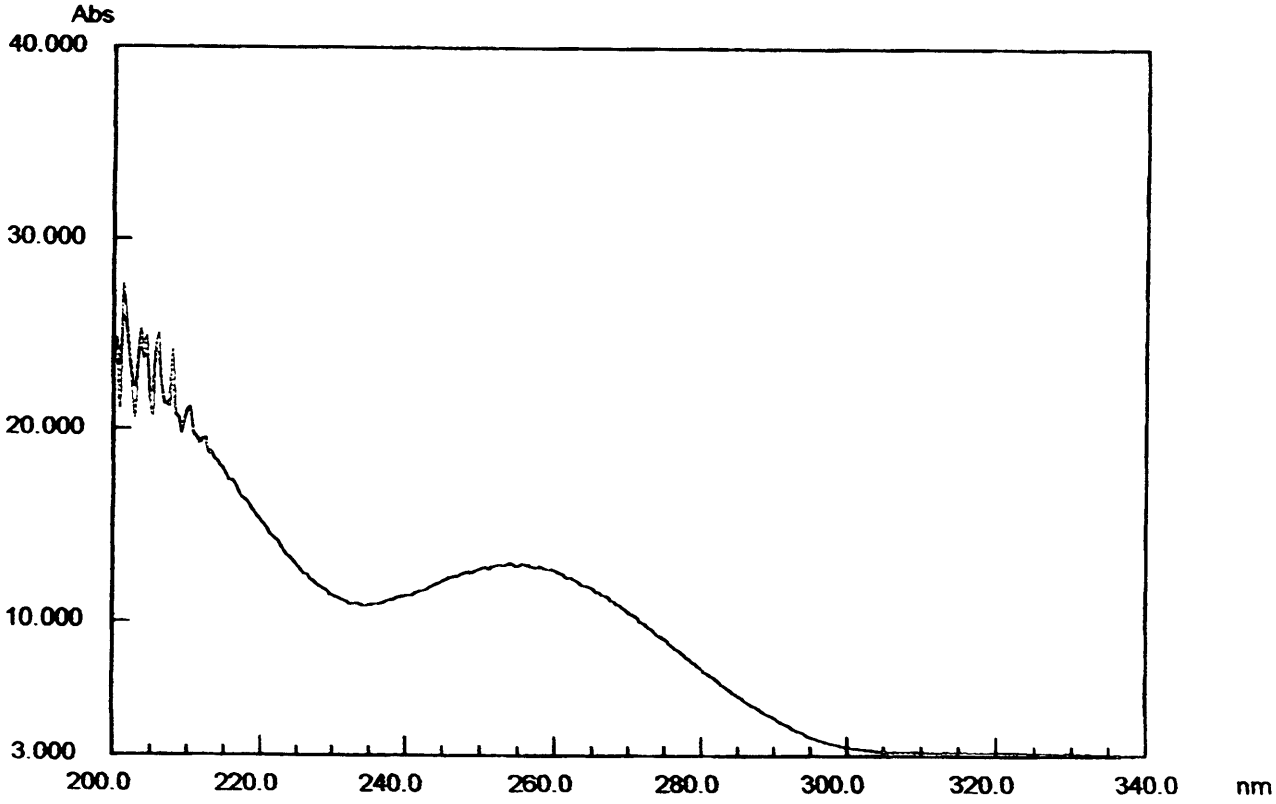
Date : 04/8/16 13:17
Sample :
Comment :

Instrument
Integration : 32

DNA Parameter
Mode : RNA
Dilution : 1.00
Background : Off
Factor : 40.0
Pure : ON
P.Conc : Off
M.Conc : Off
Unit : ug/uL

Upper : 100.0
Lower : 0.0
Expeted Value : 2.000

Report: 04/8/16 13:27




Result

| 230.0 (nm) | 260.0 (nm) | 280.0 (nm) | Ratio | Conc (ug/uL) | Pure (%) | Flag |
|---|---|---|---|---|---|---|
| 11.327 | 12.676 | 7.568 | 1.675 | 0.51 | 83.7 | |

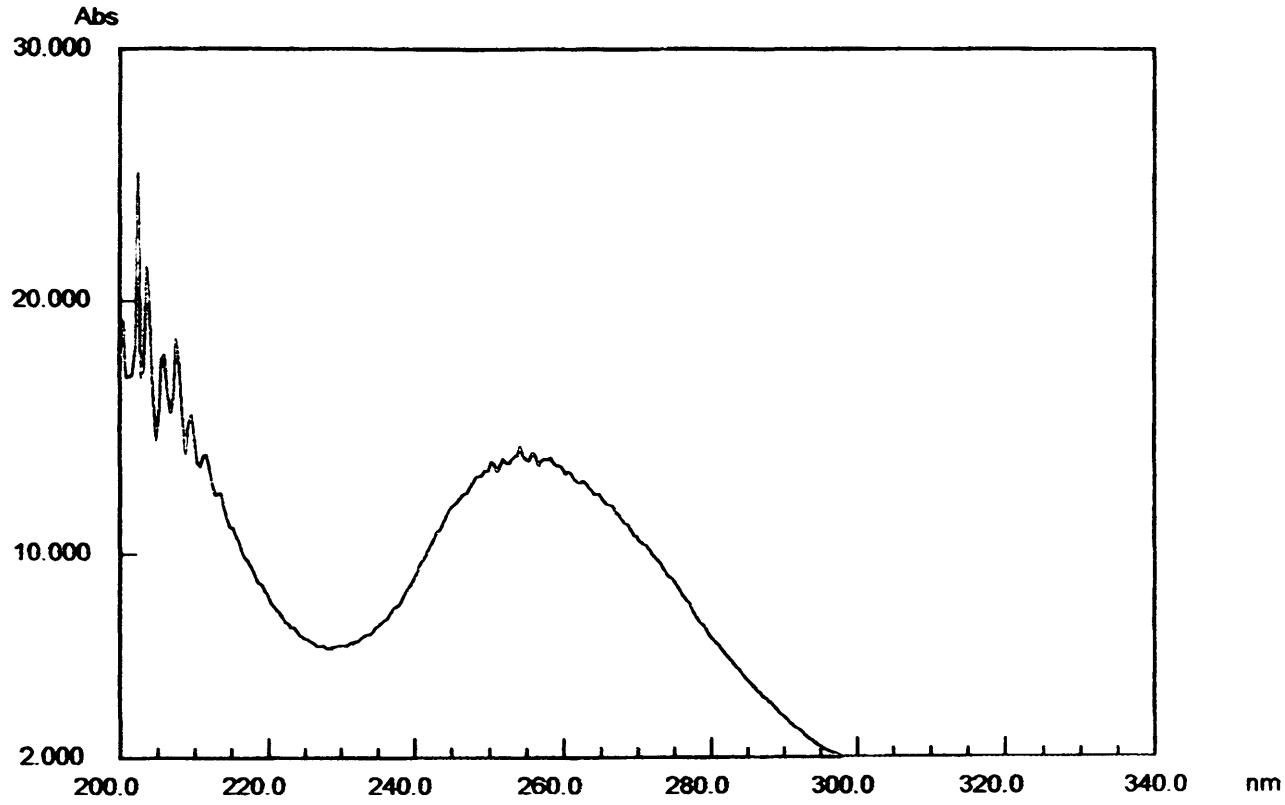
Date : 04/8/16 13:15
Sample :
Comment :

Instrument
Integration : 32

DNA Parameter
Mode : RNA
Dilution : 1.00
Background : Off
Factor : 40.0
Pure : ON
P.Conc : Off
M.Conc : Off
Unit : ug/uL

Upper : 100.0
Lower : 0.0
Expeted Value : 2.000

Report: 04/8/17 12:25




Result

| 230.0 (nm) | 260.0 (nm) | 280.0 (nm) | Ratio | Conc (ug/uL) | Pure (%) | Flag |
|---|---|---|---|---|---|---|
| 6.376 | 13.445 | 6.753 | 1.991 | 0.54 | 99.5 | |

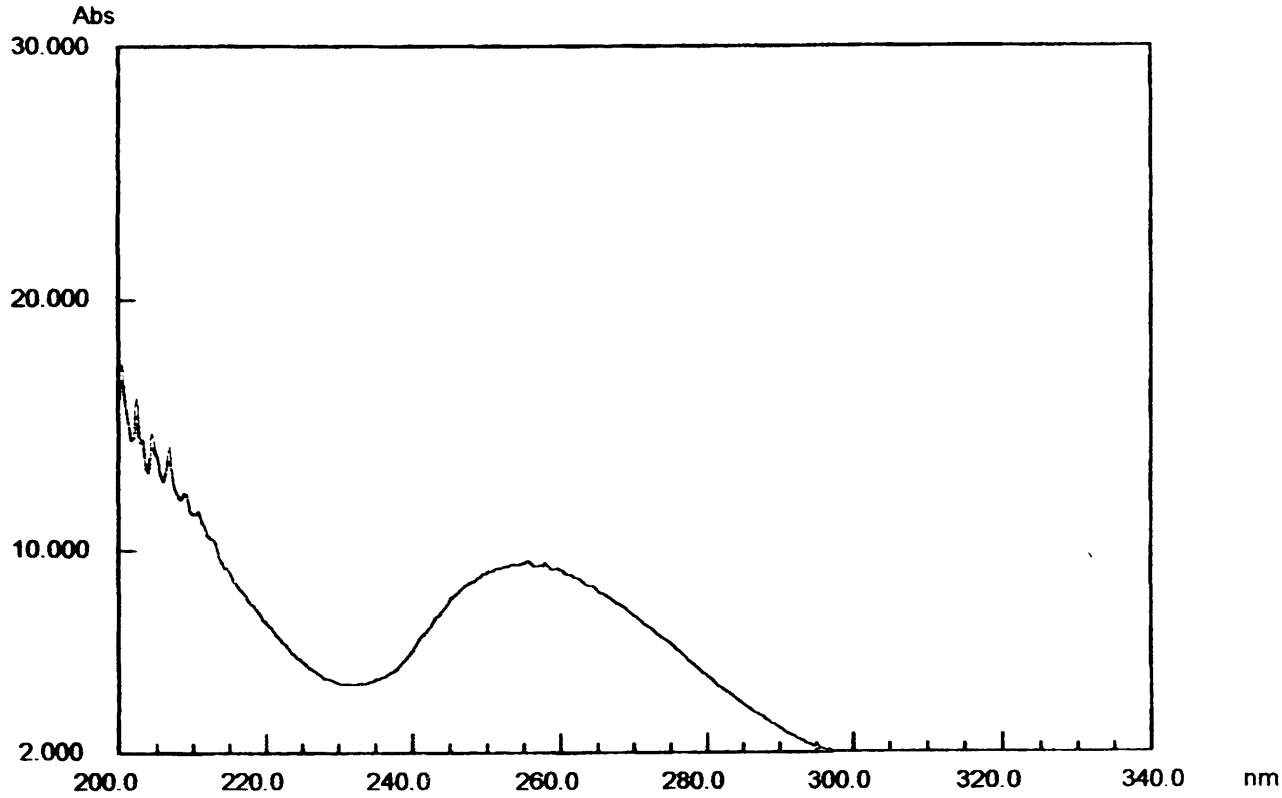
Date : 04/8/17 12:13
Sample :
Comment :

Instrument
Integration : 32

DNA Parameter
Mode : RNA
Dilution : 1.00
Background : Off
Factor : 40.0
Pure : ON
P.Conc : Off
M.Conc : Off
Unit : ug/uL

Upper : 100.0
Lower : 0.0
Expeted Value : 2.000

Report: 04/8/17 12:26




Result

| 230.0 (nm) | 260.0 (nm) | 280.0 (nm) | Ratio | Conc (ug/uL) | Pure (%) | Flag |
|---|---|---|---|---|---|---|
| 4.793 | 9.217 | 5.064 | 1.820 | 0.37 | 91.0 | |

Date : 04/8/17 12:14
Sample :
Comment :

Instrument
Integration : 32

DNA Parameter
Mode : RNA
Dilution : 1.00
Background : Off
Factor : 40.0
Pure : ON
P.Conc : Off
M.Conc : Off
Unit : ug/uL

Upper : 100.0
Lower : 0.0
Expeted Value : 2.000

# References

(1) Ajani, JA, Roth JA, Ryan B, *et al*, Evaluation of pre- and postoperative chemotherapy for respectable adenocarcinoma of the esophagus or gastroesophageal junction. J Clin Oncol,1990, 8; pp 1231-38.

(2) Dehdashti F and Barry A, Siegel *et al* Neoplasms of the oesophagus and stomach Seminars in Nuclear Medicine, 2004, Volume 34 Issue 3; pp 198-208

(3) CRC (Cancer Research Campaign). Cancer Stats Mortality - UK. 1999. London: Cancer Research Campaign.

(4) CRC (Cancer Research Campaign). Factsheet 1.1 Incidence [147] UK. 1998. London: Cancer Research Campaign.

(5) Bailey, Love *et al*, Short Practice of Surgery, 24th Edition 2004, pp 990

(6) Koop H *et al* Gastrooesophageal reflux disease and Barrett's oesophagus, Endoscopy, 2002, 34; pp. 97–103.

(7) El-Serag H. B, Mason A.C, Key C.R *et al*, Epidemiological differences between adenocarcinoma of the oesophagus and adenocarcinoma of the gastric cardia in the USA, Gut, 2002, 50; pp. 368–372.

(8) Lagergren J, Bergstrom R, Lindgren A *et al.*, Symptomatic gastrooesophageal reflux as a risk factor for oesophageal adenocarcinoma. N. Engl. J. Med, 1999, 349; pp. 825–831.

(9) Hong-Ping Yu, Xiao-Yong *et al*, DNA repair gene XRCC1 polymorphisms, smoking, and oesophageal cancer risk, Cancer Detection and Prevention, 2004 Volume 28, Issue 3, pp 194-199

(10) Zou J, Tao Z *et al*, Cancer and non-cancer epidemiological study in the high background radiation area of Yangjiang, China International Congress Series Volume 1276 , February 2005, pp 97-101

(11) Urschel JD, Vasan H, Blewett CJ. A meta-analysis of randomized controlled trials that compared neoadjuvant chemotherapy and surgery to surgery alone for resectable esophageal cancer, Am J Surg, 2002, 183; pp 274-79.

(12) Lerut T, Esophageal surgery at the end of the millennium, J Thorac Cardiovasc Surg, 1998, 116; pp 1-20.

(13) Allum WH, Griffin SM, Colin-Jones D *et al*, Guidelines for the management of oesophageal and gastric cancers. Gut, 2002, 50; (Suppl): v1-v23.

(14) Kaklamanos IG, Walker GR, Livingstone AS, Neoadjuvant treatment for respectable cancer of the esophagus and the gastroesophageal junction: a meta-analysis of randomised clinical trials, Ann Surg Oncol, 2003, 10; pp 754-61.

(15) Malthaner R, Fenlon D. Preoperative chemotherapy for resectable esophageal cancer (Cochrane Review). In The Cochrane Library, 2002, Issue 4. Oxford: Update Software.

(16) Stein HJ, Sendler A, Fink U, Siewert JR, Multidisciplinary approach to esophageal and gastric cancer, Surg Clin North Am, 2000, 80; pp 659-82.

(17) Entwistle JWC, Goldberg M, Multimodality therapy for resectable cancer of the thoracic esophagus, Ann Thorac Surg, 2002, 73; pp1009-15.

(18) Lerut T, Coosemans W, De Leyn P, Van Raemdonck D, Deneffe G, Decker G. Treatment of esophageal carcinoma, Chest,1999, 116 (suppl 6); pp 463S-465S.

(19) Bartels H, Stein HJ, Siewart JR, Preoperative risk analysis and postoperative mortality of oesophagectomy for respectable oesophageal cancer, Br J Surg,1998, 85; pp 840-44.

(20) Harrison LE. Is esophageal cancer a surgical disease? J Surg Oncol, 2000: 75, pp 227-31.

(21) Lerut T, Coosemans W, Raemdonck D. Cancer of the esophagus and gastro-esophageal junction: potentially curative therapies, Surg Oncol, 2001, 10: pp113-22.

(22) Stein HJ, Feith M, Siewart JR. Cancer of the esophago-gastric junction. Surg Oncol 2000; 9: 35-41.

(23) Wayman J, Bennett MK, Griffin SM. The pattern of recurrence of adenocarcinoma of the oesophago-gastric junction. Br J Cancer 2002; 86: pp 1223-29.

(24) Malthaner RA, Wong RK, Zuraw L; Members of the Gastrointestinal Cancer Disease Site Group of Cancer Care Ontario's Program in Evidence-based Care. Neoadjuvant or adjuvant therapy for resectable esophageal cancer: a systematic review and meta-analysis. BMC Med 2004; 2: pp 35.

(25) O'Sullivan GC, Sheehan D, Shanahan F. Micrometastases in esophagogastric cancer: high detection rate in resected rib segments. Gastroenterology 1999; 116: pp 543-48.

(26) Cancer Research UK, http//www.cancerresearch.org PO Box 123, 61, Lincoln's Inn Fields, London WC2A 3PX

(27) Sutton P, Clark P. Neo-adjuvant treatment for esophageal cancer. GI Cancer 2000; 3: pp 231-38.

(28) Walsh TN, Grannell M, Mansoor S. Predictive factors for success of neo-adjuvant therapy in upper gastrointestinal cancer. J Gastroenterol Hepatol 2002; 17 Suppl: S172-75.

(29) Zacherl J, Sendler A, Fink U. Current status of neoadjuvant therapy for adenocarcinoma of the distal esophagus. World J Surg 2003; 27: pp 1067-74.

(30) Kelsen DP, Ginsberg R, Pajak TF, et al. Chemotherapy followed by surgery compared with surgery alone for localized esophageal cancer. N Engl J Med 1998;339: pp 1979–84.

(31) Medical Research Council Oesophageal Cancer Working G. Surgical resection with or without preoperative chemotherapy in oesophageal cancer: a randomised controlled trial. Lancet 2002;359: pp1727–33.

(32) Malthaner R, Fenlon D. Preoperative chemotherapy for resectable thoracic esophageal cancer. The Cochrane Library, Cochrane Database of Systematic Reviews 2007 Issue 1

(33) Phase III Randomized Study of Neoadjuvant Cisplatin and Fluorouracil Versus Cisplatin, Epirubicin, and Fluorouracil in Patients With Resectable Adenocarcinoma of the Esophagus. http://www.nci.nih.gov/search/ViewClinicalTrials. aspx?cdrid=69457&version=Health Professional & protocol searchid= 1145210.

(34) Allum W, Cunningham D, Weeden S on behalf of the National Cancer Research Institute Upper Gastrointestinal Cancer Clinical Study Group. Perioperative chemotherapy in operable gastric and lower oesophageal cancer. A randomised controlled trial (the MAGIC trial ISRCTN 93793971). Proc Am Soc Clin Oncol 2003; 21: 998 (abstract).

(35) Webb A, Cunningham D, Wardley A, Meehan M. Randomized trial comparing epirubicin, cisplatin, and fluorouracil versus fluorouracil, doxorubicin, and methotrexate in advanced esophagogastric cancer, J Clin Oncol 1997; 15: pp 261-67.

(36) Waters JS, Norman A, Hickish T. Long-term survival after epirubicin, cisplatin and fluorouracil for gastric cancer: results of a randomized trial. Br J Cancer 1999; 80: pp 269-72.

(37) Forshaw M. J. Gossage J. A. Mason R. C, Neoadjuvant chemotherapy for oesophageal cancer: The need for accurate response prediction and evaluation, The Surgeon, December 2005 Vol 3 No 6

(38) Roth JA, Pass HI, Steinberg S. Randomised clinical trial of preoperative and postoperative adjuvant chemotherapy with cisplatin, vindesine and bleomycin for carcinoma of the esophagus, J Thorac Cardiovasc Surg 1988; 96: pp 242-48.

(39) Arnott SJ, Duncan W, Gignoux M, et al. Preoperative radiotherapy in oesophageal carcinoma: a meta-analysis using individual patient data (Oesophageal Cancer Collaborative Group). Int J Radiat Oncol Biol Phys 1998;41: pp 579–83.

(40) Launois B, Delarue D, Campion JP, Kerbaol M. Preoperative radiotherapy for carcinoma of the oesophagus. Surg Gynecol Obstet 1981;153:690–2.

(41) Wang M, Gu XZ, Zhang DW. Randomized clinical trial on the combination of preoperative irradiation and surgery in the treatment of oesophageal carcinoma: report on 206 patients. Int J Radiat Oncol Biol Phys 1989;16: pp 325–7.

(42) Gignoux M, Roussel A, Paillot B, et al. The value of preoperative radiotherapy in oesophageal cancer: results of a study of the E.O.R.T.C. World J Surg 1987;11:pp 426–32.

(43) Nygaard K, Hagen S, Hansen HS, et al. Pre-operative radiotherapy prolongs survival in operable oesophageal carcinoma: a randomised, multicenter study of pre-operative radiotherapy and chemotherapy. The second Scandinavian trial in oesophageal cancer. World J Surg 1992;16: pp1104–9

(44) Thirion P, Piedbois Y, Tierney J, et al. Combined preoperative treatment in resectable esophageal carcinoma: where do we stand? Proc Am Soc Clin Oncol 2002;21: pp133b.

(45) Urschel JD, Vasan H. A meta-analysis of randomised controlled trials that compared neoadjuvant chemoradiation and surgery to surgery alone for resectable esophageal cancer. Am J Surg 2003;185: pp 538–43.

(46) Fiorica F, Di Bona D, Schepis F, et al, Preoperative chemoradiotherapy for oesophageal cancer, a systematic review and meta-analysis, Gut 2004;53: pp 925–30

(47) Urba SG, Orringer MB, Strawderman M. Randomized trial of preoperative chemoradiation versus surgery alone in patients with locoregional oesophageal carcinoma. J Clin Oncol 2001;19:305–13. Radiation therapy and combined modality treatment of gastrointestinal carcinomas 133

(48) Walsh TN, Noonan N, Hennessy TP. A comparison of multimodal therapy and surgery for esophageal adenocarcinoma. N Engl J Med 1996;335: pp 462–7.

(49) Apinop C, Puttisak P, Preecha N. A prospective study of combined therapy in oesophageal cancer. Hepato-Gastroenterology 1994;41: pp 391–3.

(50) Burmeister BH, Smithers BM, Fitzgerald L, et al. A randomized phase III trial of preoperative chemoradiation followed by surgery (CR-S) versus surgery alone (S for localized resectable cancer of the oesophagus). Proc Am Soc Clin Oncol 2002;21:130a.

(51) Lee JL, Kim SB, Jung HY, et al. A single institutional phase III trial of preoperative chemotherapy with hyperfractionation radiotherapy plus surgery (CRT-S) versus surgery (S) alone for stage II, III resectable oesophageal squamous cell carcinoma (SCC): an interim analysis. Proc Am Soc Clin Oncol 2003;22:260.

(52) Bosset JF, Gignoux M, Triboulet JP, et al. Chemoradiotherapy followed by surgery compared with surgery alone in squamous-cell cancer of the oesophagus. N Engl J Med 1997;337: pp 161–7.

(53) Le Prise E, Etienne PL, Meunier B, et al. A randomised study of chemotherapy, radiation therapy, and surgery versus surgery for localized squamous cell carcinoma of the esophagus. Cancer 1994;73: pp 1779–84.

(54) Urba S, Orringer M, Turrisi A, Whyte R, Iannettoni M, Forastiere A. A randomised trial comparing surgery to preoperative concomittant chemoradiation plus surgery in patients with resectable esophageal cancer. Update analysis. Proc Am Soc Clin Oncol 1997;16:277a.

(55) Phase III Randomised Study of Neoadjuvant Radio-chemotherapy and Surgery Versus Surgery Alone in Patients With Resectable Thoracic Oesophageal Cancer FFCD-9901. http://www.nci.nih.gov/search/ViewClinicalTrials.aspx?cdrid=257600&version=HealthProfessional&protocolsearchid=1145210.

(56) Gibson M, Burtness B, Heath E, Heitmiller R, Forastiere AA. Effect of neoadjuvant chemoradiotherapy on pathologic stage and survival in patients with locally advanced oesophageal cancer. J Clin Oncol 2004;22(14S):4032.

(57) Brooks JA, Werner E, Jain NP, et al. A retrospective analysis to identify variables predictive of achieving a pCR after neoadjuvant chemoradiation therapy for locally advanced oesophageal cancer. J Clin Oncol 2004;22(14S):4061.

(58) Khushalani NI, McKinley BP, Petrelli NJ. Regional chemotherapy is indicated after surgical resection of colorectal metastases to the liver: a debate. J Surg Oncol 2003;82: pp 65–72.

(59) Bedenne L, Michel P, Bouche O, et al. Randomized phase III trial in locally advanced oesophageal cancer: radiochemotherapy followed by surgery versus radiochemotherapy alone (FFCD 9102). Proc Am Soc Clin Oncol 2002;21:130a.

(60) Stahl M, Stuschke M, Lehmann N, et al. Chemoradiation with and without surgery in patients with locally advanced squamous cell carcinoma of the oesophagus. J Clin Oncol 2005;23: pp 2310–7

(61) Al-Sarraf M, Martz K, Herskovic A, et al. Progress report of combined chemoradiotherapy versus radiotherapy alone in patients with oesophageal cancer: an intergroup study. J Clin Oncol 1997;15: pp 277–84.

(62) Cooper JS, Guo MD, Herskovic A, et al. Chemoradiotherapy of locally advanced oesophageal cancer: long-term follow-up of a prospective randomised trial (RTOG 85-01). Radiation Therapy Oncology Group. JAMA 1999;281: 1623–7.

(63) Minsky BD, Pajak TF, Ginsberg RJ, et al. INT 0123 (Radiation Therapy Oncology Group 94-05) phase III trial of combined-modality therapy for oesophageal cancer: highdose versus standard-dose radiation therapy. J Clin Oncol 2002;20: pp 1167–74.

(64) Wong R, Malthaner R. Combined chemotherapy and radiotherapy (without surgery) compared with radiotherapy alone in localized carcinoma of the oesophagus. The Cochrane Library, The Cochrane Database of Systematic Reviews 2007 Issue 1

(65) Gao XS, Qiao XY, Yang XR, et al. Late course accelerated hyperfractionation radiotherapy concomitant with cisplatin in patients with oesophageal carcinoma. Oncol Rep 2002;9: pp 767–72.

(66) Teniere P, Hay JM, Fingerhut A, Fagniez PL. Postoperative radiation therapy does not increase survival after curative resection for squamous cell carcinoma of the middle and lower oesophagus as shown by a multicenter controlled trial. French University Association for Surgical Research. Surg Gynecol Obstet 1991;173: pp123–30.

(67) Zieren HU, Muller JM, Staar S. Adjuvant postoperative radiation therapy after curative resection of squamous cell carcinoma of the thoracic oesophagus: a prospective randomised study. World J Surg 1995;19: pp 444–9.

(68) Fok M, Sham JS, Wong J. Postoperative radiotherapy for carcinoma of the oesophagus: a prospective, randomised controlled study. Surgery 1993;113: pp138–47.

(69) Xiao ZF, Yang ZY, Liang J, et al. Value of radiotherapy after radical surgery for oesophageal carcinoma: a report of 495 patients. Ann Thorac Surg 2003;75: pp 331–6.

(70) Ando N, Iizuka T, Ide H, et al. Surgery plus chemotherapy compared with surgery alone for localized squamous cell carcinoma of the thoracic oesophagus: a Japan Clinical Oncology Group Study – JCOG9204. J Clin Oncol 2003;21: pp 4592–6.

(71) Ando N, Iizuka T, Kakegawa T, et al. A randomized trial of surgery with and without chemotherapy for localized squamous carcinoma of the thoracic oesophagus: the Japan Clinical Oncology Group Study. J Thorac Cardiovasc Surg 1997;114: pp 205–9.

(72) Tachibana M, Yoshimura H, Kinugasa S, et al. Postoperative chemotherapy vs chemoradiotherapy for thoracic oesophageal cancer: a prospective randomised clinical trial. Eur J Surg Oncol 2003;29(Sep): pp 580–7.

(73) Phase III Randomized Study of Adjuvant Chemoradiation After Resection in Patients With Gastric or Gastroesophageal Adenocarcinoma. http://www.nci.nih.gov/search/ViewClinicalTrials.aspx?cdrid=258787&version=Health Professional & protocolsearchid=1145210.

(74) Lordick F, Stein HJ, Peschel C, Siewert JR. Neoadjuvant therapy for oesophagogastric cancer. Br J Surg 2004; 91: pp 540-51.

(75) Allum W, Cunningham D, Weeden S on behalf of the National Cancer Research Institute Upper Gastrointestinal Cancer Clinical Study Group. Perioperative chemotherapy in operable gastric and lower oesophageal cancer. A randomised controlled trial (the MAGIC trial ISRCTN 93793971). Proc Am Soc Clin Oncol 2003; 21: 998 (abstract).

(76) Geh, JI, Glynne-Jones R, Livingstone JI, Townsend ER et al. Preoperative ECF chemotherapy in Gastro-Oesophageal Adenocarcinoma. Clin Oncol (R Coll Radiol) 2000; 12: pp182-87.

(77) Medical Research Council Oesophageal Cancer Working Party. Surgical resection with or without neoadjuvant chemotherapy in oesophageal cancer: a randomised control trial. Lancet 2002; 359: 1727-33.

(78) Andreyev HJ, Norman AR, Cunningham D. Why do patients with weight loss have a worse outcome when undergoing chemotherapy for gastrointestinal malignancies. Eur J Cancer 1998. 34: pp 503-9

(79) Steyn RS, Grenier I, Matthews HR. Weight gain as an indicator of response to chemotherapy for oesophageal carcinoma. Clin Oncol (R Coll Radiol) 1995; 7: pp 382-84

(80) Brown WA, Thomas J, Smithers BM. Use of oesophagogastroscopy to assess the response of oesophageal carcinoma to neoadjuvant therapy. Br J Surg 2004; 91: pp199-204.

(81) Giovannini M, Seitz JF, Resbaut M et al. Endoscopic ultrasonography for assessment of the response to combined radiation and chemotherapy in patients with esophageal cancer. Endoscopy 1997; 29; pp 4-9.

(82) Jones DR, Detterbeck FC, Tepper JE. Induction chemoradiotherapy followed by oesophagectomy in patients with carcinoma of the esophagus. Ann Thorac Surg 1997; 64: pp 185-92.

(83) World Health Organisation. WHO handbook for reporting results of cancer treatment. 1979; publication no. 48. Geneva: WHO.

(84) Miller AB, Hoogstraten B, Staquet M, Winkler A. Reporting results of cancer treatment. Cancer 1981; 47: pp 207-14.

(85) Laterza E, de Manzoni G, Cordiano C. Endoscopic ultrasonography in the staging of esophageal carcinoma after preoperative radiotherapy and chemotherapy. Ann Thorac Surg 1999; 67: pp 1466-69.

(86) Beseth BD, Bedford R, Isacoff WH, Holmes EC, Cameron RB. Endoscopic ultrasound does not accurately assess pathologic stage of esophageal cancer after neoadjuvant chemoradiotherapy. Am Surg 2000; 66: pp 827-31.

(87) Zuccaro G Jr, Rice TW, Adelstein DJ. Endoscopic ultrasound cannot determine suitability for esophagectomy after aggressive chemoradiotherapy for esophageal cancer. Am J Gastroenterol 1999; 94: pp 906-12.

(88) Isenberg G, Chak A, Sivak MV Jr. Endoscopic ultrasound in restaging of esophageal cancer after neoadjuvant chemoradiation. Gastrointest Endosc 1998; 48: pp 158-63.

(89) Chak A, Canto MI, Sivak MV Jr. Endosonographic assessment of multimodality therapy predicts survival of esophageal carcinoma patients. Cancer 2000; 88:pp 1788-95.

(90) Willis J, Cooper GS, Chak A. Correlation of EUS measurement with pathologic assessment of neoadjuvant therapy response in esophageal carcinoma. Gastrointest Endosc 2002; 55: pp 655-61.

(91) Beyer T, Townsend DW, Nutt R. A combined PET/CT scanner for clinical oncology. J Nucl Med 2000; 41: pp1369-79.

(92) Larson SM, Schoder H, Yeung H. Positron emission tomography/computerized tomography functional imaging of esophageal and colorectal cancer. Cancer J 2004; 10: pp 243-50.

(93) Kostakoglu L, Goldsmith SJ. PET in the assessment of therapy response in patients with carcinoma of the head and neck and of the esophagus. J Nucl Med 2004; 45: pp 56-68.

(94) Lin J, Beerm DG. Molecular biology of upper gastrointestinal malignancies. Semin Oncol 2004; 31: pp 476-86.

(95) Metzger R, Leichman C.G. Danenberg K.D et al., ERCC1 mRNA levels complement thymidylate synthase mRNA levels in predicting response and survival for gastric cancer patients receiving combination cisplatin and fluorouracil chemotherapy. J. Clin. Oncol. 16 (1998), pp. 309–316.

(96) François E, Marc Ychou et al, Combined radiotherapy, 5-fluorouracil continuous infusion and weekly oxaliplatin in advanced rectal cancer: A phase I study, European Journal of Cancer, Volume 41, Issue 18, December 2005, pp 2861-2867

(97) Lawes D and Taylor I, Chemotherapy for colorectal cancer—an overview of current management for surgeons, European Journal of Surgical Oncology, Volume 31, Issue 9, November 2005, pp 932-941

(98) Oehler C, Ciernik F I, Radiation therapy and combined modality treatment of gastrointestinal carcinomas, Cancer Treatment Reviews, Volume 32, Issue 2, 2006, pp119-138,

(99) Geh IJ, Bond SJ, Glynne-Jones R, Systematic overview of preoperative (neoadjuvant) chemoradiotherapy trials in oesophageal cancer: Evidence of a radiation and chemotherapy dose response. Radiotherapy and Oncology, 2006, Volume 78, Issue 3, March, pp 236-244

(100) Duhaylongsod FG, Gottfried MR, Wolfe WG. The significance of c-erb B-2 and p53 immunoreactivity in patients with adenocarcinoma of the esophagus. Ann Surg 1995; 221: pp 677-83.

(101) Walsh TN, Grannell M, Mansoor S. Predictive factors for success of neo-adjuvant therapy in upper gastrointestinal cancer. J Gastroenterol Hepatol 2002; 17 Suppl: S172-75.

(102) Pozzo C, Barone C,Bugat R. Irinotecan in combination with 5-fluorouracil and folinic acid or with cisplatin in patients with advanced gastric or esophageal-gastric junction adenocarcinoma: results of a randomized phase II study. Ann Oncol 2004; 15: pp 1773-81.

(103) Ajani JA, Walsh G, Roth JA. Preoperative induction of CPT-11 and cisplatin chemotherapy followed by chemoradiotherapy in patients with locoregional carcinoma of the esophagus or gastroesophageal junction. Cancer 2004; 100: pp 2347-54.

(104) Lordick F, von Schilling C, Peschel C. Phase II trial of irinotecan plus docetaxel in cisplatin-pretreated relapsed or refractory oesophageal cancer. Br J Cancer 2003; 89: pp 630-33.

(105) Slater S, Shamash J, Slevin ML. Irinotecan, cisplatin and mitomycin in inoperable gastro-oesophageal and pancreatic cancers - a new active regimen. Br J Cancer 2002; 87: pp 850-53.

(106) Ajani JA, Baker J, Pisters PW, Charnsangavej C. CPT-11 plus cisplatin in patients with advanced, untreated gastric or gastroesophageal junction carcinoma: results of a phase II study. Cancer 2002; 94: pp 641-46.

(107) Conroy T, Etienne PL, Wils J; European Organisation for Research and Treatment of Cancer Gastrointestinal Tract Cancer Cooperative Group. Vinorelbine and cisplatin in metastatic squamous cell carcinoma of the oesophagus: response, toxicity, quality of life and survival. Ann Oncol 2002; 13: pp 721-29.

(108) Einzig AI, Neuberg D, Benson AB 3rd. Phase II trial of docetaxel (Taxotere) in patients with adenocarcinoma of the upper gastrointestinal tract previously untreated with cytotoxic chemotherapy: the Eastern Cooperative Oncology Group (ECOG) results of protocol E1293. Med Oncol 1996; 13: pp 87-93.

(109) Khushalani NI, Leichman CG, Leichman L. Oxaliplatin in combination with protracted-infusion fluorouracil and radiation: report of a clinical trial for patients with esophageal cancer. J Clin Oncol 2002; 20: pp 2844-50.

(110) Assersohn L, Brown G, Norman AR. Phase II study of irinotecan and 5-fluorouracil/leucovorin in patients with primary refractory or relapsed advanced oesophageal and gastric carcinoma. Ann Oncol 2004; 15: pp 64-69

(111) Ozols R.F. and Williams S., Testicular cancer. Curr. Probl. Cancer 1989, 13; pp. 285–335

(112) Loehrer P.J. and Einhorn L., Drugs five years later: Cisplatin. Ann. Intern. Med. 1984, 100; pp. 704–713

(113).Ozols R.F, Ovarian cancer. Curr. Probl. Cancer, 1992,16; pp. 61–126

(114) Kavanagh J.Tresukosol D, Edwards C et al., Carboplatin reintroduction after taxane in patients with platinum-refractory epithelial ovarian cancer. J. Clin. Oncol. 1995, 13; pp. 1584–1588.

(115) Misset J L, Bleiberg H et al, Oxaliplatin clinical activity: a review Critical Reviews in Oncology/Hematology, 2000, Volume 35, Issue 2; pp 75-93

(116) Rixe O, Ortuzar W, Alvarez M. et al., Oxaliplatin, tetraplatin, cisplatin, and carboplatin: spectrum of activity in drug-resistant cell lines and in the cell lines of the National Cancer Institute's Anticancer Drug Screen panel. Biochem. Pharmacol. 1996, 52; pp. 1855–1865

(117) Chollet P, Bensmaine A, Brienza S et al., Single agent activity of oxaliplatin in heavily pretreated advanced epithelial ovarian cancer. Ann. Oncol, 1996, 7; pp.1065–1070

(118) Soulié P, Bensmaine A, Garrino C et al., Oxaliplatin/cisplatin (L-OHP/CDDP) combination in heavily pretreated ovarian cancer. Eur. J. Cancer, 1997, 33; pp. 1400–1406.

(119) Al Batran SE, Atmaca A, Hegewisch-Becker S, et al. Phase II trial of biweekly infusional fluorouracil, folinic acid, and oxaliplatin in patients with advanced gastric cancer. J Clin Oncol 2004; 22: pp 658–663.

(120) Chao Y, Yeh KH, Chang CJ, et al. Phase II study of weekly oxaliplatin and 24-h infusion of high-dose 5-fluorouracil and folinic acid in the treatment of advanced gastric cancer. Br J Cancer 2004; 91: pp 453–458.

(121) Louvet C, Andre T, Tigaud JM, et al. Phase II study of oxaliplatin, fluorouracil, and folinic acid in locally advanced or metastatic gastric cancer patients. J Clin Oncol 2002; 20: pp 4543–4548.

(122) Saltz LB, Cox JV, Blanke C, Moore MJ, et al. Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer. Irinotecan Study Group. N Engl J Med 2000; 343:905–14.

(123) Cunningham D, Glimelius B. A phase III study of irinotecan (CPT-11) versus best supportive care in patients with metastatic colorectal cancer who have failed 5-fluorouracil therapy. V301 Study Group. Semin Oncol 1999;26: pp 6–12.

(124) De Gramont A, Figer A, Hmissi A, Cassidy J, et al. Leucovorin and fluorouracil with or without oxaliplatin as first-line treatment in advanced colorectal cancer. J Clin Oncol 2000;18: pp 2938–47.

(125) Tournigand C, Andre T, Mignard D, et al. FOLFIRI followed by FOLFOX6 or the reverse sequence in advanced colorectal cancer: a randomized GERCOR study. J Clin Oncol 2003.

(126) Rosenberg B, Van Camp L. and Krigas T, Inhibition of cell division in Escherichia coli by electrolysis products from platinum electrode. Nature, 1965, 205 pp. 698–699

(127) Wiltshaw E and Carr B, cis-Platinum (II)diammine-dichloride. In: T.A. Connors and I.J. Roberts, Editors, Platinum Coordination Complexes in Cancer Chemotherapy, Springer-Verlag, Heidelberg (1974), pp. 178–182

(128) Higby D.J, Wallace H.J, Holland J.F, Diaminodichloroplatinum: a phase I study showing responses in testicular and other tumors. Cancer, 1974, 33; pp. 1219–1225.

(129) Higby D.J, Wallace H.J, Holland J.F, Diamminodichloroplatinum in the chemotherapy of testicular tumors. J. Urol. 1974, 112; pp. 100–104

(130) Wiltshaw E, Subramarian, S, Barker H, Cancer of the ovary: a summary of experience with cis- dichlorodiammineplatinum (II) at the Royal Marsden Hospital. Cancer Treat. Rep. 1979, 63; pp 1545–1548

(131) O'Dwyer P J, Stevenson J.P and Johnson S.W, Clinical pharmacokinetics and administration of established platinum drugs, Drugs, 2000, 59 (Suppl. 4); pp. 19–27

(132) Gralla R.J, Itri L.M, Braun,Jr D.W, Antiemetic efficacy of high-dose metoclopramide: randomized trials with placebo and prochlorperazine in patients with chemotherapy-induced nausea and vomiting. N. Engl. J. Med. 1981, 305; pp 905–909.

(133) Glabbeke M. van, Renard J, Sessa C, Iproplatin and carboplatin induced toxicities: overview of phase II clinical trial conducted by the EORTC Early Clinical Trials Cooperative Group (ECTG), Eur J Cancer Clin Oncol, 1988, 24; pp. 255–262

(134) Kweekel D.M, Gelderblomb H *et al,* Pharmacology of oxaliplatin and the use of pharmacogenomics to individualize therapy, Cancer Treatment Reviews, 2005, Volume 31, Issue 2; pp 90-105

(135) Desoize B, and Madoulet C, Particular aspects of platinum compounds used at present in cancer treatment, Critical Reviews in Oncology/Hematology, 2002, Volume 42, Issue 3, pp 317-325

(136) Levi F, Metzger G, Massari C *et al*, Oxaliplatin: pharmacokinetics and chronopharmacological aspects. Clin. Pharmacokinet, 2000, 38; pp 1–21.

(137) Boughattas N A , Levi F, Fournier C, Circadian rhythm in toxicities and tissue uptake of 1,2-diamminocyclohexane(trans-1)oxalatoplatinum(II) in mice. Cancer Res. 1989, 49; pp. 3362–3368.

(138) Caussanel J.P, Levi F, Brienza S, Phase I trial of 5-day continuous venous infusion of oxaliplatin at circadian rhythm-modulated rate compared with constant rate. J. Natl. Cancer Inst. 1990, 82; pp 1046–1050

(139) de Gramont A , Bosset J.F, Milan C, Randomized trial comparing monthly low-dose leucovorin and fluorouracil bolus with bimonthly high-dose leucovorin and fluorouracil bolus plus continuous infusion for advanced colorectal cancer: a French intergroup study. J. Clin. Oncol, 1997, 15; pp.808–815.

(140) Maindrault-Goebel F, Louvet C, Andre T, Oxaliplatin added to the simplified bimonthly leucovorin and 5-fluorouracil regimen as second-line therapy for metastatic colorectal cancer (FOLFOX6). GERCOR. Eur. J. Cancer, 1999, 35; pp. 1338–1342.

(141) Seitz J F et al. Adenocarcinoma of the distal oesophagus and gastric cardia what chemotherapy or chemotherapy for recurrent or metastatic disease. Cancer Radiotherapy, 2001, 5;(Suppl 1): pp 107-112.

(142) Reed E, Cisplatin and analogs. In: B.A. Chabner and D.L. Longo, Editors, Cancer Chemotherapy and Biotherapy: Principles and Practice, Lippincott Williams and Wilkins, Philadelphia, 2001, pp. 447–465.

(143) Culy C.R, Clemett D and Wiseman L R, Oxaliplatin, A review of its pharmacological properties and clinical efficacy in metastatic colorectal cancer and its potential in other malignancies. Drugs, 2000, 60; pp. 895–924.

(144) O'Dwyer P.J, Johnson S.W.and Hamilton T.C, Cisplatin and its analogues. In: V.T. DeVita, S. Hellman and S.A. Rosenberg, Editors, Cancer: Principles and Practice of Oncology, Lippincott-Raven, Philadelphia, 1997, pp. 418–432.

(145) Brabec V and Leng, M., 1993. DNA interstrand cross-links of trans-diamminedichloroplatinum (II) are preferentially formed between guanine and complementary cytosine residues. Proc. Natl. Acad. Sci. U.S.A. 90, pp. 5345–5349

(146) Go R.S and Adjei A A, Review of the comparative pharmacology and clinical activity of cisplatin and carboplatin. J. Clin. Oncol, 1999, 17; pp. 409–422

(147) Pinto A.L and Lippard S.J, Binding of the antitumour drug cis-diamminedichloroplatinum (II) (cisplatin) to DNA. Biochim. Biophys. Acta, 1985, 780; pp. 167–180.

(148) Pascoe J.M and Roberts J.J, Interactions between mammalian cell DNA and inorganic platinum compounds: II. Interstrand cross-linking of isolated and cellular DNA by platinum (IV) compounds. Biochem. Pharmacol, 1974, 23; pp. 1345–1357.

(149) Saris CP, Van de Vaart P, Begg AC. DNA adduct formation in vitro and in platinum sensitive and resistant cells after treatment with lobaplatin, oxaliplatin and cisplatin. Seventh International Symposium on Platinum. Amsterdam, 1995, O42; (abstract).

(150) Faivre S, Raymond E, Chapman W, Lesion in cellular DNA and apoptosis induced by oxaliplatin, Proc Am Assoc Cancer Res 1997 (abstract).

(151) McHugh P J, Spanswick V J, and Hartley J A, Repair of DNA interstrand crosslinks: molecular mechanisms and clinical relevance. THE LANCET Oncology, 2001, 2; pp 73-80

(152) Zwelling L.A, Michaels S, Kohn K.W, DNA cross-linking as an indicator of sensitivity and resistance of mouse L1210 leukemia to cis-diamminedichloroplatinum (II) and L-phenylalanine mustard. Cancer Res, 1981, 41; pp. 640–649.

(153) Plooy A.C, Dijk M V and Lohman P H, Induction and repair of DNA cross-links in chinese hamster ovary cells treated with various platinum coordination compounds in relation to platinum binding to DNA, cytotoxicity, mutagenicity, and antitumor activity. Cancer Res.1984, 44; pp. 2043–2051

(154) Jennerwein M M, Eastman A and Khokhar A, Characterization of adducts produced in DNA by isomeric 1,2-diaminocyclohexaneplatinum (II) complexes. Chem. Biol. Interact, 1989, 70; pp. 39–49

(155) Faivre S, Chan D *et al*, DNA strand breaks and apoptosis induced by oxaliplatin in cancer cells Biochemical Pharmacology, 2003, Volume 66, Issue 2, pp 225-237

(156) Szymkowski D.E, Yarema K, Essigmann J.M, An intrastrand d(GpG) platinum crosslink in duplex M13 DNA is refractory to repair by human cell extracts. Proc. Natl. Acad. Sci. USA, 1992, 89; pp. 10772–10776

(157) Mello J.A, Lippard S.J and Essigmann J.M, DNA adducts of cis-diamminedichloroplatinum(II) and its trans isomer inhibit RNA polymerase II differentially in vivo. Biochemistry, 1995, 34; pp 14783–14791

(158) Hector S, Bolanowska-Higdon W, Pendyala L, In vitro studies on the mechanisms of oxaliplatin resistance. Cancer Chemother. Pharmacol, 2001, 48; pp. 398–406.

(159) Saris C P, Van de Vaart P.J.M and Blommaert F.A, In vitro formation of DNA adducts by cisplatin, lobaplatin and oxaliplatin in calf thymus DNA in solution and in cultured human cells, Carcinogenesis, 1996, 17; pp. 2763–2769.

(160) Arnould S, Hennebelle I, Guichard S, Cellular determinants of oxaliplatin sensitivity in colon cancer cell lines, Eur J of Cancer, 2003, 39 issue I; pp 112-119

(161) Dunkern R, Fritz G and Kaina B, Cisplatin-induced apoptosis in 43-3B and 27-1 cells defective in nucleotide excision repair, Mutat. Res 2001, 486; pp. 249–258.

(162) Page J D, Husain I and Chaney S G, Effect of the diaminocyclohexane carrier ligand on platinum adduct formation, repair, and lethality, Biochemistry, 1990, 29; pp. 1016–1024

(163) Reardon J T, Vaisman A, Chaney S G, Efficient nucleotide excision repair of cisplatin, oxaliplatin, and Bis-aceto-ammine-dichloro-cyclohexylamine-platinum (IV) (JM216) platinum intrastrand DNA diadducts. Cancer Res, 1999, 59; pp. 3968–3971

(164) Woynarowski J M, Faivre S, Herzig M.C.S, Oxaliplatin-induced damage of cellular DNA. Mol. Pharmacol, 2000, 58; pp. 920–927

(165) Woynarowski J.M, Chapman W.G, Juniewicz P, Sequence- and region-specificity of oxaliplatin adducts in naked and cellular DNA. Mol. Pharmacol.1998, 54; pp. 770–777.

(166) Walker P.R and Sikorska M, New aspects of the mechanism of DNA fragmentation in apoptosis, Biochem. Cell Biol, 1997, 75; pp. 287–299

(167) Cummings B.S and Schnellmann R.G, Cisplatin-induced renal cell apoptosis: caspase-3-dependent and -independent pathways, J. Pharmacol. Exp. Ther, 2002, 302; pp. 8–17.

(168) Suo Z, Lippard S.J and Johnson K.A, (GpG)/cis-diammineplatinum(II) adduct-induced inhibition of DNA polymerisation, Biochemistry, 1999, 38; pp 715–726.

(169) Zamble D.B and Lippard S.J, Cisplatin and DNA repair in cancer chemotherapy, Trends Biochem. Sci, 1995, 20 pp. 435–439

(170) Luo Y L Z and Bregman D B, RNA polymerase II large subunit is cleaved by caspases during DNA damage-induced apoptosis Biochemical and Biophysical Research Communications, 2002, Volume 296; pp 954-961

(171) Hoeijmakers J H, Genome maintenance mechanisms for preventing cancer. Nature, 2001, 411; pp. 366–374

(172) Luo Z, Ultraviolet radiation alters the phosphorrlation of RNA polymerase II large subunit and accelerate its proteasome dependent degradation. Mutations Research, 2001, 486; pp, 259-274

(173) Cullinane C, Inhibition of RNA polymerase ii Transcription in human cell extracts by Cisplatin DNA damage. Biochemistry, 1999, 38; pp 6204-6212

(174) Ljungman M F, Zhang F and McKay B.C, Inhibition of RNA polymerase II as a trigger for the p53 response. Oncogene, 1999, 18; pp. 583–592

(175) Petersen L N, Mamenta E L, Bohr V A, Increased gene specific repair of cisplatin induced interstrand crosslinks in cisplatin resistant cell lines, and studies on carrier ligand specificity, Carcinogenesis, 1996, 17; pp 2597 Section 1.01

(176) Hamilton T and Johnson S, Abstracts 8th international symp, Platinum and other metal coordination compounds in cancer chemotherapy, 1999, 3.01.

(177) Schmidt W, Role of carrier ligand in platinum resistance of human carcinoma cell lines, Cancer Research, 1993, 53; pp 799.

(178) Chaney S G and Vaisman A, Specificity of Platinum –DNA adduct repair, Journal of Inorganic Biochemistry, 1999, 77; pp 71-81

(179) Naegeli H, Roadblocks and detours during DNA replication: mechanisms of mutagenesis in mammalian cells, Bioassay, 1994, 16; pp 557

(180) Gibbons G R, Kaufmann W K, Chaney S G, Role of DNA replication in carrier-ligand-specific resistance to platinum compounds in L1210 cells carcinogenesis, 1991, 12, pp 2253, Section 1.01

(181) Mamenta E L, Poma E E, Chaney S G, Enhanced replicative bypass of platinum-DNA adducts in cisplatin-resistant human ovarian carcinoma cell lines Cancer Res, 1994, 54, pp 3500

(182) Judson I and Kelland L.R, New developments and approaches in the platinum arena, Drugs, 2000, 59, pp 29–36

(183) Aaltonen L, Peltomaki P, Leach FS, Clues to the pathogenesis of familial colorectal cancer, Science 1999, 260:812–6

(184) Chaney S, Sancar A. DNA repair: enzymatic mechanisms and relevance to drug response, J Natl Cancer Inst 1996; 88:1346–60

(185) Eastman A, Mechanisms of resistance to cisplatin, Cancer Treat. Res, 1991, 57; pp 233–249.

(186) Inoue T, Ishida T, Sugio K, Glutathione-S-transferase Pi is a powerful indicator in chemotherapy of human lung squamous-cell carcinoma, Respiration, 1995, 62; pp 223–227.

(187) Johnson SW, Ferry K V and Hamilton T C, Recent insights into platinum drug resistance in cancer, Drug Resist Updates, 1998, 1; pp 243–254

(188) Sharp S Y, O'Neill C F, Kelland L R, Retention of activity by the new generation platinum agent AMD0473 in four human tumour cell lines possessing acquired resistance to oxaliplatin, European Journal of Cancer, 2002, 38; pp 2309-2315

(189) El-akawi Z, Abu-hadid M, Perez R, .Altered glutathione metabolism in oxaliplatin resistant ovarian carcinoma cells, Cancer Lett, 1996, 105 pp 5–14

(190) Sartorius U A and Krammer P H, Upregulation of Bcl-2 is involved in the mediation of chemotherapy resistance in human small cell lung cancer cell lines, Int. J. Cancer, 2002, 97; pp 584–592

(191) Alas S, Ng C P and Bonavida B, Rituximab modifies the cisplatin-mitochondrial signaling pathway, resulting in apoptosis in cisplatin-resistant non-Hodgkin's lymphoma. Clin Cancer Res, 2002, 8; pp 836–845

(192) Han J Y, Lee K S, The relationship between cisplatin-induced apoptosis and p53, Bcl-2 and Bax expression in human lung cancer cells. Korean J. Int. Med. 1999, 14, pp 42–52

(193) Gourdier I, Pau B, Drug specific resistance to oxaliplatin is associated with apoptosis defect in a cellular model of colon carcinoma, FEBS Letters, 2002, 529 pp 232-236

(194) Qin L F and Ng I O, Induction of apoptosis by cisplatin and its effect on cell cycle-related proteins and cell cycle changes in hepatoma cells, Cancer Lett, 2002, 175; pp. 27–38

(195) Akiyama Z S, Chen T, Resistance to cisplatin, Anticancer Drug Des, 1999, 14; pp 143–151

(196) Andrews P A, Velury S and Howell S B, cis- diamminedichloroplatinum (II) accumulation in sensitive and resistant human ovarian carcinoma cells, Cancer Research, 1998, 48; pp 68-73

(197) Levi F, Mishima M, Oxaliplatin: pharmacokinetics and chronopharmacological aspects, Clin Pharmacokinet, 2000, 38; (5), pp 1–21

(198) Di Francesco A M, Ruggiero A and Riccardi R, Cellular and molecular aspects of drugs of the future: oxaliplatin, Cell Mol Life Sci, 2002, 59; pp 1914–1927

(199) Graham M A, Lockwood G F, Greenslade D, Clinical pharmacokinetics of oxaliplatin: a critical review. Clin. Cancer Res, 2000, 6; pp 1205–1218

(200) Calvert A H and Egorin M J, Carboplatin dosing formulae: gender bias and the use of creatinine-based methodologies, Eur J Cancer, 2002, 38; pp 11–16

(201) Woynarowska B A, MacDonald J R, Differential cytotoxicity and induction of apoptosis in tumour and normal cells by hydroxymethylacylfulvene (HMAF), Biochem. Pharmacol, 2000, 59; pp 1217–1226

(202) Herzig M C, Woynarowski J M, Irofulven induces apoptosis in breast cancer cells regardless of caspase-3 status. Breast Cancer Res Treat, 2002, 71; pp 133–143.

(203) Chabner B A, Roberts Jr TG, Timeline: chemotherapy and the war on cancer. Nat Rev Cancer 2005, 5: pp 65–72.

(204) DeVita Jr VT, Hellman S, Rosenberg SA, Cancer principles and practice in oncology, Seventh edition, Lippincott Williams and Wilkins 2005.

(205) Efferth T, Manfred Pharmacogenetics for individualized cancer chemotherapy, Pharmacology & Therapeutics, 2005, 107; pp 155 – 176

(206) Robert J, Vekris A, Bonnet J, Predicting drug response based on gene expression, Crit Rev Oncol/Hematol 2004; 51: pp 205–27.

(207) Robert J, Morvan V L, Bonnet J, Predicting drug response and toxicity based on gene polymorphisms. Crit Rev Oncol/Hematol 2005, 54: pp 171–96

(208) Evgeny N, Imyanitov a, Moiseyenko M, Molecular-based choice of cancer therapy, Realities and expectations, Clinica Chimica Acta, 2007, 379; pp 1–13

(209) Takebayashi P, Pourquier D B, Antiproliferative activity of ecteinascidin 743 is dependent upon transcription-coupled nucleotide-excision repair, Nat. Med, 2001, 7; pp 961–966

(210) Zamble D B, Lippard S J, Cisplatin and DNA repair in cancer chemotherapy, Trends Biochem. Sci, 1995, 20; pp 435–439

(211) Zlatanova J, Yaneva J, Leuba S H, Proteins that specifically recognize cisplatin-damaged DNA: a clue to anticancer activity of cisplatin, FASEB J. 1998, 12; pp 791–799

(212) Larminat F, Bohr V A, Role of the human ERCC-1 gene in gene-specific repair of cisplatin-induced DNA damage, Nucl Acids Res, 1994, 22; pp 3005–3010

(213) Koberle B, Masters J R, Wood R D, Defective repair of cisplatin-induced DNA damage caused by reduced XPA protein in testicular germ cell tumours, Curr Biol, 1999, 95; pp 273–276

(214) Damia G, Imperatori L, Sensitivity of CHO mutant cell lines with specific defects in nucleotide excision repair to different anti-cancer agents, Int. J. Cancer, 1996, 66; pp 779–783

(215) Dabholkar M, Vionnet J, Reed E, Messenger RNA levels of XPAC and ERCC1 in ovarian cancer tissue correlate with response to platinum-based chemotherapy, J Clin Invest, 1994, 94: pp 703–708

(216) Codegoni A M, Broggini M, Expression of genes of potential importance in the response to chemotherapy and DNA repair in patients with ovarian cancer, Gynecol Oncol , 1997, 65 1; pp 130–137

(217) Damia G, Guidi G, Expression of genes involved in nucleotide excision repair, and sensitivity to cisplatin and melphalan in human cancer cell lines, Eur J Cancer, 1998, 34 11; pp 1783–1788

(218) Moorehead R.A, Armstrong S G, Nucleotide excision repair in the human ovarian carcinoma cell line (2008) and its cisplatin-resistant variant (C13), Cancer Chemother Pharmacol, 1996, 38 3; pp 245–253

(219) Shirota Y, Stoehlmacher J, Brabender J, ERCC1 and thymidylate synthase mRNA levels predict survival for colorectal cancer patients receiving combination oxaliplatin and fluorouracil chemotherapy, J Clin Oncol, 2001;19: pp 4298-304

(220) Katherine V, Johnson S W, Increased Nucleotide Excision Repair in Cisplatin-Resistant Ovarian Cancer Cells ROLE OF ERCC1–XPF, Biochemical Pharmacology, 2000, 60; pp 1305–1313

(221) Andrews P A, Velury S, Howell S B, cis-Diamminedichloroplatinum(II) accumulation in sensitive and resistant human ovarian carcinoma cells, Cancer Res 1988, 48: pp 68–73

(222) Kuppen PJ, Schrier PI, cis-Diamminedichloroplatinum(II)-resistant sublines derived from two human ovarian tumor cells, Cancer Res,1988, 48: pp 3355–3359

(223) Kelland L R, Harrap K R, Mechanism-related circumvention of acquired cis-diamminedichloroplatinum(II) resistance using two pairs of human ovarian carcinoma cell lines by ammine/amine platinum(IV) dicarboxylates, Cancer Res, 1992, 52: pp 3857–3864

(224) Schilder R J, Hall L, Hamilton T C, Metallothionein gene expression and resistance to cisplatin in human ovarian cancer, Int J Cancer, 1990, 45: pp 416–422

(225) Behrens B C, Hamilton T C, Ozols R F, Characterization of a cis-diamminedichloroplatinum(II)-resistant human ovarian cancer cell line and its use in evaluation of platinum analogues, Cancer Res,1987, 47: pp 414–418

(226) Mistry P, Kelland L R, Harrap K R, The relationships between glutathione, glutathione-S-transferase and cytotoxicity of platinum drugs and melphalan in eight human ovarian carcinoma cell lines. Br J Cancer, 1991, 64: pp 215–220

(227) Hamilton T C, Ozols RF, Augmentation of adriamycin, melphalan, and cisplatin cytotoxicity in drug-resistant and -sensitive human ovarian cancer cell lines by buthionine sulfoximine mediated glutathione depletion, Biochem Pharmacol, 1985, 34: pp 2583–2586

(228) Godwin A K, Meister A, Anderson M E, High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase oglutathione synthesis. Proc Natl Acad Sci USA, 1992, 89: pp 3070– 3074

(229) Masuda H, Ozols R F, Hamilton T C, Increased DNA repair as a mechanism of acquired resistance to cis-diamminedichloroplatinum(II) in human ovarian cancer cell lines, Cancer Res 1988, 48: pp 5713–5716

(230) Lai G-M, Ozols R F, Hamilton T C, Enhanced DNA repair and resistance to cisplatin in human ovarian cancer, Biochem Pharmacol, 1988, 37: pp 4597–4600

(231) Masuda H, Tanaka, Kusaba I, Increased removal of DNA-bound platinum in a human ovarian cancer cell line resistant to cis-diamminedichloroplatinum(II), Cancer Res,1990, 50: pp 1863–1866

(232) Parker R J, Eastman A, Reed E, Acquired cisplatin resistance in human ovarian cancer cells is associated with enhanced repair of cisplatin-DNA lesions and reduced drug accumulation, J Clin Invest, 1991, 87: pp 772–777

(233) Johnson S W, Perez R P, Hamilton T C, Role of platinum-DNA adduct formation and removal in cisplatin resistance in human ovarian cancer cell lines. Biochem Pharmacol, 1994, 47: pp 689–697

(234) Zhen W, Link C J Jr, Bohr V A, Increased gene-specific repair of cisplatin interstrand cross-links in cisplatin-resistant human ovarian cancer cell lines, Mol Cell Biol, 1992, 12: pp 3689–3698

(235) Johnson SW, Laub PB, Hamilton T C, Increased platinum-DNA damage tolerance is associated with cisplatin resistance and cross-resistance to various chemotherapeutic agents in unrelated human ovarian cancer cell lines, Cancer Res 1997, 57: pp 850–856

(236) Hamaguchi K, Godwin A K, Hamilton T C, Cross-resistance to diverse drugs is associated with primary cisplatin resistance in ovarian cancer cell lines, Cancer Res 1993, 53: pp 5225–5232

(237) Rosell R, Cecere F, Taron M, Predicting the outcome of chemotherapy for lung cancer, Current Opinion in Pharmacology, 2006, 6:pp 323–331

(238) Rosell R, Cobo M, Etxaniz O, Applications of genomics in NSCLC, Lung Cancer 2005, 50: pp S33-S40.

(239) Lord RVN, Brabender J, Gandara D, Low ERCC1 expression correlates with prolonged survival after cisplatin plus gemcitabine chemotherapy in non-small cell lung cancer, Clin Cancer Res, 2002;8: pp 2286-91

(240) Rosell R et al, Molecular markers and targeted therapy with novel agents: prospects in the treatment of non-small cell lung cancer, Lung Cancer 2002, 38; pp S43-S49.

(241) Hockenbery D, Nuñez G, Korsmeyer S J, Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature, 1990, 348; pp 334

(242) Borner C, The Bcl-2 protein family: sensors and checkpoints for life-or-death decisions. Mol. Immunol, 2003, 39; pp 615–647

(243) Katja C, Green D R, The machinery of programmed cell death. Pharmacology and Therapeutics, 2001, 92; pp 57-70

(244) Ledgerwood E C, Pober J S and Bradley J R, Recent advances in the molecular basis of TNF signal transduction, Lab. Invest, 1999, 79; pp 1041–1050

(245) Polyak K, Xia Y, Vogelstein B, A model for p53-induced apoptosis, Nature, 1997, 389; pp 300–305

(246) Zornig M, Busch G, Beneke R, Survival and death of prelymphomatous B-cells from N-myc/bcl-2 double transgenic mice correlates with the regulation of intracellular Ca2+ fluxes, Oncogene, 1995, 11; pp 2165

(247) Hacki J, Egger L, Apoptotic crosstalk between the endoplasmic reticulum and mitochondria controlled by Bcl-2, Oncogene, 2000, 19; pp 2286

(248) Dunkern T R, Kaina B, Cisplatin-induced apoptosis in 43-3B and 27-1 cells defective in nucleotide excision repair, Mutation Research, 2001, 486; pp 249–258

(249) Larminat F, Bohr V A, Role of the human ERCC-1 gene in gene-specific repair of cisplatin-induced DNA damage, Nucl. Acids Res, 1994, 22; pp 3005–3010

(250)Lips j, Kaina B, DNA double-strand breaks trigger apoptosis in p53 deficient fibroblasts, Carcinogenesis, 2001, 22; pp 579–585

(251) Miyake H, Hanada N, Saya H, Overexpression of Bcl-2 in bladder cancer cells inhibits apoptosis induced by cisplatin and adenoviral-mediated p53 gene transfer, Oncogene, 1998, 16; pp 933–943

(252) Mese H, Sasaki A, Matsumura T, Regulation of apoptosis reduction in the cisplatinresistant A431 cell line by Bcl-2 and CPP32, Chemotherapy, 2000, 46; pp 69–76.

(253) Li S, Zhao Y, Relief of extrinsic pathway inhibition by the Bid-dependent mitochondrial release of Smac in Fas-mediated hepatocyte apoptosis, J Biol Chem 2002, 277; pp 26912–26920

(254) Bargou C, Wagener C, Overexpression of the death-promoting gene bax-alpha which is downregulated in breast cancer restores sensitivity to different apoptotic stimuli and reduces tumor growth in SCID mice. J. Clin. Invest, 1996, 97; pp 2651

(255) Beerheide W, Tan Y J, Downregulation of proapoptotic proteins Bax and Bcl-X(S) in p53 overexpressing hepatocellular carcinomas, Biochem. Biophys. Res. Commun, 2000, 273, pp 54

(256) Strasser A, Huang D C and Vaux D L, The role of the bcl-2/ced-9 gene family in cancer and general implications of defects in cell death control for tumourigenesis and resistance to chemotherapy, Biochim. Biophys. Acta, 1997, 1333; pp F151

(257) Johnstone R W, Ruefli A A and Lowe S W, Apoptosis: a link between cancer genetics and chemotherapy, Cell, 2002, 108 pp 153

(258) Panaretakis T, Pokrovskaja K, Grander D, Activation of Bak, Bax, and BH3-only proteins in the apoptotic response to doxorubicin, J. Biol. Chem, 2002, 277 pp 44317

(259) Shi Y et al, Mechanisms of caspase activation and inhibition during apoptosis, Mol. Cell, 2002, 9; pp 459–470

(260) Van der Craan M, Declercq W, Vandenabeele P, The proteolytic procaspase activation network: an in vitro analysis, Cell Death Differ, 1999, 6; pp 1117–1124

(261) Kidd V J, Lahti J M Teitz T, Proteolytic regulation of apoptosis, Cell Dev. Biol, 2000, 11; pp 191–201

(262) Zeuner A, Eramo A, De Matia R, Caspase activation without death, Cell Death Differ, 1999, 6, pp 1075–1080

(263) Narita M, Shimizu S, Ito T, Tsujimoto Y, Bax interacts with the permeability transition pore to induce permeability transition and cytochrome c release in isolated mitochondria, Proc. Natl. Acad. Sci. U.S.A, 1998, 25; pp14681–14686

(264) Bratton G B, MacFarlane M, Cohen G M, Protein complexes activate distinct caspase cascades in death receptor and stress-induced apoptosis, Exp. Cell Res, 2000, 256; pp 27–33

(265) Myashita T, Reed J C, Tumour suppressor p53 is a direct transcriptional activator of the human Bax gene, Cell, 1995, 80; pp 293–299

(266) Miyashita T, Harigari M, Reed J C, Identification of a p53-dependent negative response element in the bcl-2 gene, Cancer Res, 1994, 54; pp 3131–3135

(267) Lee H, Larner J M, Hamlin J I, Cloning and characterization of Chinese hamster p53 cDNA, Gene, 1997, 184; pp 177–183

(268) Wesselborg S, Engels I H, Schulze- Osthoff K, Anticancer drugs induce caspase-8/FLICE activation and apoptosis in the absence of CD95 receptor/ligand interaction, Blood, 1999, 93; pp 3053–3063

(269) Ferreira C G, Tolis C, Giaccone G, Drug-induced apoptosis in lung cancer cells is not mediated by the Fas/FasL (CD95/APO1) signalling pathway, Clin. Cancer Res, 2000, 6; pp 203–212

(270) Belka C, Heinrich V, Budach W, Ionizing radiation and the activation of caspase-8 in highly apoptosis-sensitive lymphoma cells, Int. J. Radiat. Biol, 1999, 75; pp 1257–1264

(271) AlamarBlue™ information booklet from BioSource International, Inc., USA, available on website http//www.biosource.com

(272) Fields R D, Lancaster M V, Dual-attribute continuous monitoring of cells proliferation/cytotoxicity. Am. Biotechnol. Lab, 1993, 11(4): pp 48-50

(273) Nociari M M, Shalev A, Russo C, A novel one-step, highly sensitive fluorometric assay to evaluate cell-mediated cytotoxicity, J. Immunol. Meth, 1998, 213 (2): pp 157-167

(274) Pagé B, Pagé M, Noël C, A new fluorimetric assay for cytotoxicity measurements in vitro, Internat. J. Oncol. 1993, 3: 473-479

(275) Desaulniers D K, Leingartner T Z, and Foster W G, Optimization of a MCF-E3 cell proliferation assay and effects of environmental pollutants and industrial chemicals. Toxicol. In Vitro, 1998, 12: pp 409-422

(276) Ling F C, Finkensieper A, Wartenberg M, Hölscher A H and Schneider P M, Z Gastroenterol 2005; pp 43

(277) AlamarBlue™ information booklet from Serotec product datasheet, available on website http//www.serotec.com

(278) Adapted from Roche Diagnostics website 2005, http//www.roche.com

(279) Granchi D, Giunti A, Bone cement extracts modulate the osteoprotegerin/osteoprotegerin-ligand expression in MG63 osteoblast-like cells, Biomaterials, 2002, 23; pp 2359-2365

(280) Information booklet provided by the QIAGEN™ for RNA extraction, Boundary court, Gatwick Rd, Crawley, West Sussex, RH10 9AX

(281) Hansen M B, Nielsen S E and Berg K, Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill, J Immunol Methods, 1989, 119: pp 203–210

(282) Manley J L, Fire A, Gefter M L, DNA-dependent transcription of adenovirus genes in a soluble whole-cell extract, Proc Natl Acad Sci, 1980, 77: pp 3855–3859

(283) Britten R.A, Liu D, Murray D et al, ERCC1 expression as a molecular marker of cisplatin resistance in human cervical tumour cells, Int. J. Cancer 89 (2000) 453–457

(284) Li Q, Yu J J, Mu C, Association between the level of ERCC-1 expression and the repair of cisplatin-induced DNA damage in human ovarian cancer cells, Anticancer Res, 2000, 20; pp 645–652

(285) Li L, Peterson C A, Legerski R J, Mutations in XPA that prevent association with ERCC1 are defective in nucleotide excision repair. Mol. Cell Biol, 1995, 15; pp 1993–1998

(286) Azuma M, Sato M et al, Cisplatin induces apoptosis in oral squamous carcinoma cells by the mitochondria-mediated but not the NF-kB-suppressed pathway, Oral Oncology, 2003, 39; pp 282–289

(287) Gelasco A Lippard S J, NMR solution structure of a DNA dodecamer duplex containing a cis-diammineplatinum(II) adduct d(GpG) intrastrand cross-link, the major adduct of the anticancer drug cisplatin, Biochemistry, 1998, 37; pp 9230–9239
(288) Gelasco A, Lippard S J, Anticancer activity of cisplatin and related complexes. In: Clarke, M.J., Sadler, P.J. (Eds.), Metallopharmaceuticals I. DNA Interactions. Springer, Berlin, 1999 pp 1–43

(289) Takahara P M, Rosenzweig A C, Lippard S J, Crystal structure of double-stranded DNA containing the major adduct of the anticancer drug cisplatin, Nature 1995, 377; pp. 649–652

(290) Petersen L N, Mamenta E L, Bohr V A, Increased gene specific repair of cisplatin induced interstrand crosslinks in cisplatin resistant cell lines, and studies on carrier ligand specificity, Carcinogenesis, 1996, 17; pp 2597 Section 1.01

(291) Yarema K J, Wilson J M, Essigmann, J M, Effects of DNA adduct structure and distribution on the mutagenicity and genotoxicity of two platinum anticancer drugs J, Mol Biol, 1994, 236; pp 1034